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UNIVERSIDADE DE COIMBRA

The multifaceted role of the endocannabinoid system in the regulation of cerebral glucose uptake

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Attila Köfalvi no Laboratório de Neuromodulação e Metabolismo do Centro de Neurociências e Biologia Celular (CNC) e da Professora Doutora Emília Duarte da Faculdade de Ciências e Tecnologia, Universidade de Coimbra.

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2013

Agradecimentos/ Acknowledgements

I would like to first acknowledge to my postgraduate supervisor Doctor Attila Köfalvi for taking me through this difficult task of educational research. I am deeply grateful for the effort, cooperation and patient in spite of the difficult challenges and predicaments that we were faced with. Thank you for all the teaching and the stimulus to improve my qualities and overcome my weaknesses.

I would like to express my great appreciation to my co-supervisor Doctor Emilia Duarte.

I wish to thank to my lab partner Carla Santos, for basically everything in this year. Thank you for the friendship, cooperation and patience.

I also would like to thank to the members of Neuromodulation and Metabolism group and Purine group, for all the help and sharing of scientific knowledge. Cristina, I am grateful for the months we spent together developing "our" technique! Thank you for helping me and for teaching me. I am also deeply grateful to Doctor Rodrigo Cunha for providing me the opportunity to spend a period in his lab.

Gostaria de agradecer à minha família, em especial aos meus pais e ao meu irmão, por toda a ajuda, suporte e encorajamento ao longo destes anos. Obrigada por acreditarem nas minhas capacidades, por me apoiarem e confiarem no meu percurso.

Para todos os meus amigos de Amarante e Braga, que me acompanharam durante estes anos todos, obrigada por todo o suporte e conforto mesmo estando separados. Obrigada por me incentivarem a continuar a lutar para ultrapassar todas as etapas. Obrigada pela partilha de conhecimento e conselhos.

Um muito obrigada aos meus amigos de Coimbra porque sem vocês estes dois anos não teriam sido tão incrivéis. E aos que me ajudaram nos últimos meses desta etapa, especialmente ao Tiago, um obrigada especial porque não há palavras para descrever o quanto agradeço a vossa ajuda.

Table of contents

List of figures IV

Abstract VI

Resumo VIII

Abbreviation list XI

Chapter 1. Introduction 1

1.1. Historical introduction to the endocannabinoid system (ECS) 1

1.2. The ECS and its physiological role 1

1.2.1. The endocannabinoids (eCBs) 1

1.2.2. Cannabinoid receptors 3

1.2.3. Cannabinoid receptors pharmacology 5

1.3. The pathophysiological roles of the ECS 5

1.3.1. Metabolic control of ECS 5

1.4. Cerebral glucose metabolism 6

1.5. Insulin 7

1.6. Diseases of impaired insulin signalling 9

1.6.1. Diabetes type 1, 2 and 3 9

1.6.2. Glucocorticoid-induced insulin resistance 11

1.7. Cannabinoid receptors and insulin 12

1.8. The reward system and insulin 13

Chapter 2. Objectives 15

Chapter 3. Materials and Methods 16

3.1. Animals 16

3.1.1. Experimental model of diabetes 17

3.1.2. Fasted rats 17

3.2. In vitro glucose uptake assays 18

3.2.1. Protein quantification by the bicinchoninic acid method 19

3.3. Fluorescent glucose uptake assays in mice coronal brain slices 20

3.4. Data presentation and statistical analysis 22

3.5. Chemicals 23

Chapter 4. Results 24

4.1. CB_1R mediation of insulin-induced glucose uptake in the NAc of wild-type rats 24

4.1.1. Insulin and CB_1R blockade increased accumbal glucose uptake 24

4.1.2. Dexamethasone impairs insulin's action - an effect reversed by CB_1R blockade 25

4.1.3. The inhibition of endocannabinoid synthesis or metabolism affects insulin's action on accumbal glucose uptake 26

4.1.4. Insulin effects in glucose uptake in acute accumbal slices from fed, fasted and STZ-induced diabetic rats 27

4.2. CB_2R activation triggers glucose uptake in the hippocampus 29

4.2.1. CB_2R activation increased glucose uptake in both wild-type and TgAPP mice 29

4.2.2. Glucose uptake in other rodent strains and its regulation by CB_2R 30

4.2.3. CB_2R activation rapidly enhances fluorescent glucose uptake in hippocampal slices of young adult C57Bl/6 mice 31

Chapter 5. Discussion 33

Chapter 6. Conclusions 43

References 44

List of figures

Figure 1. The red circle hightlight the endocannabinoids synthesis/metabolism and their retrograde action in the presynaptic receptors________________________**3**

Figure 2. Schematic representation of the uptake of the radioactive glucose analog, the non-metabolizable ^{3}H-2-deoxyglucose________________________________**19**

Figure 3. Schematic representation explaining the timeline of the experiments __**21**

Figure 4. Representative graph of the real-time monotorization of fluorescence intensity changes____**22**

Figure 5. The pharmacology of insulin effect on glucose uptake, in 450 µm-thick accumbal slices of male Wistar rats, under the blockade (O-2050, 500 nM) or activation (WIN, 500 nM) of CB_1R as well as the blockade of IGF1R (OMe-Tyr, 1 µM)____**25**

Figure 6. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices of male Wistar rats, in the presence of dexamethasone (DEX, 10 µM) alone or in combination with the CB_1R antagonist, O-2050 (500 nM)____**26**

Figure 7. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices of male Wistar rats, in the presence of inhibitors of 2-AG synthesis: THL (10 µM) or metabolism: JZL184 (1 µM) and WWL70 (1 µM)____**27**

Figure 8. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices. Two concentrations of insulin were tested (30 nM and 300 nM) in fed, fasted and STZ-injected diabetic rats________________________________**28**

Figure 9. The CB_2R effect on glucose uptake in 300 µm-thick acute hippocampal slices of WT or TgAPP mice. CB_2R was activated by JWH133 (1 µM) or WIN55212-2 (1 µM) and inhibited by AM630 (1 µM); COX-2 was blocked by DuP697 (500 nM)

and FAAH and FLAT were inhibited by LY2183240 (100 nM)____29

Figure 10. Basal and JWH133-stimulated glucose uptake values in acute hippocampal slices of young adult Wistar rats (young adult Wistar rats 18-days sham and 18-days after Aβ injection (yellow bars) and another set of young adult Wistar rats (green bars)) and of young adult CD-1 mice (purple bars)____30

Figure 11. Time-course and subregional variation of the effect of CB_2R agonists on the uptake of the fluorescent glucose analogue 2-NBDG, in 300 µm-thick hippocampal slices of young C57Bl/6j male mice___________________________31

Figure 12. Time-course and fluorescence intensity variations of the effect of CB_2R agonists on the uptake of 2-NBDG, in 300 µm-thick hippocampal slices of young C57Bl/6j male mice. **A)** Changes in 2-NBDG uptake rate after the treatment with the vehicle DMSO (0.1%), GP1a (100 nM) or JWH133 (1 µM). **B)** Scatter graph illustrates the individual amplitude variations in fluorescence intensity upon treatment with DMSO, JWH133 and GP1a____32

Figure 13. Schematic diagram of the proposed model for the DEX-induced insulin-resistance.____36

Figure 14. Schematic diagram of the hypothetic glucoregulator control of anandamide in healthy rodents and under β-amyloidosis.____________________40

Abstract

Background and aims: The endocannabinoid system affects energy balance and glucose metabolism through the central control of feeding behaviour. The reward area, nucleus accumbens (NAc) is implicated in postprandial satiety and termination of food-seeking behaviour. Hence, in this study we aimed at understanding whether insulin affects accumbal activity (measured indirectly by monitoring changes in glucose uptake in accumbal slices of the rat). Furthermore, we tested if the cannabinoid receptor type-1 (CB_1R) influences insulin's observed actions. Moreover, glucocorticoid excess can lead to insulin resistance, diabetes or Alzheimer's disease, and recent findings of ours and others suggest that glucocorticoids can trigger endocannabinoid release. Thus, we also investigated the action of the glucocorticoid dexamethasone (DEX) on the insulin-mediated glucose uptake in the nucleus accumbens and whether the effect was dependent on local CB_1R signaling.

After the CB_1R, a second, so-called type-2 cannabinoid receptor (CB_2R) has been cloned, and its possible cerebral roles are also gaining support. Brain glucose hypometabolism is a preclinical symptom of Alzheimer's disease and CB_2Rs are upregulated in AD patients and in animal models of AD. Thus, we also aimed at mapping the possible involvement of CB_2Rs in hippocampal glucoregulation.

Methods: We performed these experiments in accumbal slices of Wistar rats and in hippocampal slices of wild-type rodents or AD-induced rodent models. A recently optimized *in vitro* technique was used to study glucose uptake in acute brain slices. This technique allows the measurement of the accumulation of a non-metabolizable radioactive glucose analog, ^3H-^{2}-deoxyglucose in bathed brain slices. In addition, we used a fluorescent glucose uptake assay, permitting the real-time measurement of the uptake of the fluorescent glucose analog 2-NBDG in superfused mouse hippocampal slices.

Results and discussion: Insulin (300 nM) triggered the uptake of glucose in the accumbal slices suggesting that insulin is capable of activating this reward area. CB_1R activation either by an exogenous agonist or by the inhibition of endocannabinoid degradations impaired insulin's action. DEX also prevented insulin from stimulating glucose uptake. Furthermore, insulin's action in the presence of DEX was rescued by

either the blockade of 2-AG synthesis with tetrahydrolipstatin or by the blockade of CB_1R with O-2050. These altogether suggest that dexamethasone causes accumbal insulin resistance employing the stimulation of 2-AG synthesis, resulting in CB_1R activation. Insulin probably mediates some of the postprandial satiety responses, thus the impairment of insulin actions can lead to an overfeeding behavior. In sum, CB_1R blockers would be beneficial to control food intake to prevent many cases of obesity and the consequent diabetes.

We also found that the activation of CB_2Rs increased hippocampal glucose uptake in both wild-type (WT) and human amyloid-precursor protein expressing transgenic mice (TgAPP) mice. Interestingly, the inhibition of COX-2, a metabolizing enzyme for anandamide, increased glucose uptake only in the WT mice, which is explained by that our collaborators found low anandamide levels in the hippocampi of TgApp mice when compared to the WT mice. Altogether, this indicates that anandamide through the activation of the CB_2R is capable of stimulating hippocampal glucose uptake. This should prompt additional studies to test if CB_2R agonists can be beneficial in AD via the stimulation of glucose uptake.

Altogether, our results highlight that selective targeting cannabinoid receptors or enzymes of endocannabinoid synthesis/metabolism can be a precious strategy to control diseases related with impaired brain metabolism.

Keywords: endocannabinoid system, cerebral glucose uptake, insulin, diabetes, Alzheimer’s disease, nucleus accumbens, hippocampus

Resumo

Introdução e objetivos: O sistema endocanabinóide afecta o balanço energético e o metabolismo da glicose através do controlo central do comportamento alimentar. Na área da recompensa, o núcleo accumbens (NAc) está implicado na saciedade pós-prandial e na finalização do comportamento doentio de procura alimentar. Assim, neste estudo tivemos como objetivo estudar os efeitos da insulina na actividade do NAc (medição indirecta pela monitorização das alterações da captação de glicose em fatias frescas de NAc provenientes de ratos *Wistar*). Adicionalmente, testámos se o receptor canabinóide tipo 1 (RCB_1) influencia as acções previamente observadas da insulina. O excesso de glucocorticóides pode originar insulino-resistência, diabetes ou doença de Alzheimer (DA). Tendo também em conta estudos recentes, nossos inclusive, que sugerem que os glucocorticóides desencadeiam a libertação de endocanabinóides, também investigámos a acção de um glucocorticóide, a dexametasona (DEX), na capação de glicose mediada pela insulina no NAc e se esta acção era dependente da sinalização local do receptor $CB_{1.}$
Posteriormente ao receptor CB_1, foi clonado um segundo receptor chamado de receptor canabinóide tipo 2 (RCB_2), tendo a sua função no cérebro vindo a ser explorada. O hipometabolismo da glicose no cérebro é um sintoma pré-clínico da DA e o receptor CB_2 está presente em níveis superiores tanto em pacientes como em modelos animais com DA. Deste modo, também investigámos o possível envolvimento dos receptores CB_2 na glicoregulação do hipocampo.

Métodos: As experiências foram efectuadas em fatias de NAc frescas de ratos *Wistar* e em fatias frescas de hipocampo provenientes de roedores normais ou com a indução de DA. Utilizámos uma técnica *in vitro* recentemente optimizada para estudar a captação de glicose em fatias cerebrais frescas. Esta técnica permite a medição da acumulação de um análogo de glicose radioactivo não metabolizável, a *^{3}H-^{2}-deoxyglucose,* em incubação com fatias cerebrais. Adicionalmente, usámos um ensaio de captação de glicose fluorescente, permitindo a medição em tempo real da captação do análogo de glicose fluorescente (2-NBDG) em fatias de hipocampo num sistema de perfusão.

Resultados e discussão: A insulina (300 nM) induziu a captação da glicose nas fatias de NAc, sugerindo a capacidade da insulina de ativar a área da recompensa. A ativação do receptor CB_1 por agonistas exógenos ou pela inibição da degradação de endocanabinóides prejudica a acção da insulina. A DEX também preveniu a estimulação da captação de glicose pela insulina. Verificámos também que a acção da insulina na presença de DEX foi recuperada não só pelo bloqueio da síntese de 2-AG com tetrahidrolipstatina, mas também pelo bloqueio do receptor CB_1 com O-2050. Este resultados sugerem que a DEX causa insulino-resistência no NAc e estimula a síntese de 2-AG, resultando na activação do receptor CB_1. Possivelmente, a insulina estará a mediar algumas das respostas de saciedade pós-prandial, sendo consequência da sua acção deficitária um comportamento alimentar exacerbado. Em suma, o bloqueio do RCB_1 poderá ser benéfico no controlo da ingestão de comida e por consequência casos de obesidade e diabetes.

Descobrimos também que a activação dos RCB_2 aumentou a captação de glicose no hipocampo tanto em murganhos normais como transgénicos (TgAPP) que expressam o precursor amilóide humano. Surpreendentemente, a inibição da enzima COX-2 que metaboliza a anandamida, aumentou a captação de glicose apenas nos ratos normais. A explicação surge por estudos que demonstraram que os murganhos possuem menores níveis de anandamida no hipocampo, quando comparados com ratos normais. Em conjunto, estes resultados indicam que a anandamida através da activação do RCB_2 é capaz de estimular a captação de glicose no hipocampo. Isto deve potenciar estudos futuros adicionais que testem se agonistas do RCB_2 são benéficos na DA, através da estimulação da captação de glicose.

Em conjunto, os nossos resultados elucidam que a manipulação selectiva dos receptores dos endocanabinóide ou de enzimas envolvidas na sua síntese/metabolismo podem ser uma estratégia para controlar doenças relacionadas com um metabolismo cerebral alterado.

Palavras-chave: Sistema endocanabinóide, captação cerebral de glicose, insulina, diabetes, doença de Alzheimer, núcleo accumbens e hipocampo.

Abbreviation list

2-AG, 2-arachidonoylglycerol

2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose

^{3}HDG, ^{3}H-2-deoxyglucose or 2-^{3}H(N)-deoxy-D-glucose

^{18}FDG, ^{18}F-deoxyglucose

AA, arachidonic acid

Aβ, β-amyloid

AD, Alzheimer's disease

ATP, adenosine triphosphate

AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide

ANOVA, analysis of variance

BBB, blood-brain barrier

BCA, bicinchoninic acid

BSA, bovine serum albumin

CB_1R, cannabinoid receptor type-1

CB_2R, cannabinoid receptor type-2

CBD, cannabidiol

CNS, central nervous system

COX-2, cyclooxygenase 2

DA, dopamine

DAG, diacylglycerol

DAGLα or β, DAG lipase α or β

Δ^{9} -THC, Δ^{9}-tetrahydrocannabinol

DEX, dexamethasone

DMSO, dimethyl sulfoxide

dpm, disintegration per minute

DuP697, 5-bromo-2[4-fluorophenyl]-3-[4-methylsulfonylphenyl]-thiophene

eCB, endocannabinoid

ECS, endocannabinoid system

EH, endogenous hypercortisolism

ERK, extracellular-signal-regulated kinase

FAA, fatty acid amide

FAAH, fatty acid amide hydrolase

FLAT, FAAH-like anandamide transporter

GABA, γ-aminobutyric acid

Gp1a, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-*c*]pyrazole-3-carboxamide

GPCR, G protein-coupled receptor family

GPR55, G protein-coupled receptor 55

GPR119, G protein-coupled receptor 119

GR, glucocorticoid receptor

GSK-3β, glycogen synthase kinase-3β

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPA, hypothalamic-pituitary-adrenal

IGF, insulin-like growth factor

IGF1R, insulin-like growth factor type-1 receptor

IR, insulin receptor

IRS, insulin receptor substrates

JWH133, (6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran

JZL184, 4-[*bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester

KH, Krebs'- HEPES

LTD, long-term depression

MAG, monoacylglycerol

MAGL, monoacylglycerol lipase

MAPK, mitogen-activated protein kinase

MHC, major histocompatibility complex

MR, mineralocorticoid receptor

NAc, nucleus accumbens

NADA, N-arachidonyl dopamine

NAPE-PLD, N-acylphosphatidyl-ethanolamine-specific phospholipase D

NAPE, N-arachidonoyl-phosphatidylethanolamine

NPY, neuropeptide Y

O-2050, (6aR,10aR)-1-hydroxy-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran

OMe-Tyr, I-Ome-Tyrphostin AG 538 or α-cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3',4'-dihydroxyphenyl)ketone

PET, positron-emission tomography

PI_3K, phosphoinositide-3 kinase

PIP 2 and 3, phosphatidylinositol (4,5)-bisphosphate and (3,4,5)-triphosphate

PKB, protein kinase B (or Akt)

PLCβ, phospholipase Cβ

PTGS2, prostaglandin-endoperoxide synthase 2

PUFAs, long-chain polyunsaturated fatty acids

RTK, receptor tyrosine kinase

SEM, standard error of the mean

STZ, streptozotocin or 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose

TCA, tricarboxylic acid

TgAPP, transgenic mice mutant amyloid precursor protein

THL, tetrahydrolipstatin

$TRPV_1R$, transient receptor potential Na+/Ca2+ channel subfamily vanilloid type 1

VTA, ventral tegmental area

WIN or WIN55212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

WT, wild-type

WWL70, *N*-methyl-*N*-[[3-(4-pyridinyl)phenyl]methyl]-4'-(aminocarbonyl)[1,1'-biphenyl]-4-yl carbamic acid ester

Chapter 1. Introduction

1.1. Historical introduction to the endocannabinoid system (ECS)

The plant *Cannabis sativa*, also known as marijuana or hemp has been used for at least 5000 years for recreational, religious, spiritual and medicinal purposes (Nagy et al. 2008).

The major psychoactive lipophilic constituent of the cannabis plant is Δ^9-tetrahydrocannabinol (Δ^9-THC) which was identified in the 60's (Gaoni & Mechoulam 1964), shortly after the isolation of cannabidiol (CBD) (Mechoulam & Shvo, 1963), which is one of the more than 66 biologically active components of cannabis. Many of the physiopharmacological actions of cannabis are thanked to these two molecules (De Petrocellis & Di Marzo 2009).

Since the late 80's, investigations regarding the mechanism of action of Δ^9-THC led to the discovery of a new, broad signalling system, the so-called endocannabinoid system (ECS).

The first THC-specific receptor, named cannabinoid receptor type-1 (CB_1R) was identified by Devane et al. (1988) and cloned by Matsuda et al. (1990) followed by the characterization of a second receptor, termed as cannabinoid receptor type-2 (CB_2R) (Munro et al. 1993). This latter receptor was identified by homology cloning of the CB_1R and it turned out to have 68% homology in the transmembrane domain amino acid sequence and 44% overall sequence identity to the CB_1R (Munro et al. 1993).

These discoveries opened the way to the identification of the endogenous cannabinoid ligands; among them the most characterized endocannabinoids are anandamide (arachidonoylethanolamide or AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura et al. 1995).

1.2. The ECS and its physiological role

1.2.1. The endocannabinoids (eCBs)

The two “major” eCBs, anandamide and 2-AG, are long-chain polyunsaturated fatty acids (PUFAs) and are both arachidonic acid (AA) derived lipids, although anandamide is a member of the fatty acid amide (FAA) family, whereas 2-AG is a member of the

monoacylglycerol (MAG) family (Kogan & Mechoulam 2006). They are synthesized from cell membrane phospholipids. N-arachidonoyl-phosphatidylethanolamine (NAPE) and diacylglycerols (DAGs) together with AA, are the major biosynthetic precursors of anandamide and 2-AG, respectively (Muccioli 2010). NAPE is produced from the transfer of AA from the *sn*-1 position of phospholipids to the nitrogen atom of phosphatidylethanolamine (Cadas et al. 1997), and it is directly converted into anandamide by the catalysing enzyme N-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD) (Okamoto et al. 2004).

In contrast, 2-AG is formed from the sequential hydrolysis of *sn*-2-arachidonic acid (AA)-containing DAG membrane phospholipids by first phospholipase Cβ (PLCβ) and subsequently, mostly by DAG lipase α (DAGLα), and in about 10-20% of the cases, by β (DAGLβ) (Di Marzo 2009; Best and Regehr, 2010).

On the other hand, AEA and 2-AG are degraded through hydrolysis. AEA is primarily hydrolysable by the fatty acid amide hydrolase (FAAH) which results in arachidonic acid and ethanolamine, while 2-AG is degraded by the monoacylglycerol lipase (MAGL) resulting in arachidonic acid and glycerol (Muccioli 2010).

Moreover, ECs may undergo oxidative metabolism mediated by prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2; PTGS2; COX-2). This leads to the formation of several biologically active prostaglandin-ethanolamides from AEA and prostaglandin-glycerol esters from 2-AG (Kozak et al. 2004; Di Marzo 2009).

Upon stimulation such as $[Ca^{2+}]_i$ rise and receptor-triggered $G_{q/11}$ activation alone or in combination, eCBs are "on-demand" synthesized within the cell membranes and are immediately released (De Petrocellis et al. 2004). Due to their high lipophilicity, they are not stored in the interior of synaptic vesicles as other classical neurotransmitters. Following their release, eCBs target the same cannabinoid receptors (CB_1R and CB_2R) as Δ^9-THC. In the brain, eCBs release occurs mostly post-synaptically and to a smaller extent in glial cells. Post-synaptically released endocannabinoids reach the synaptic cleft to act in a retrograde form at CB_1Rs to inhibit both excitatory and inhibitory neurotransmitter release (Figure 1) (Best & Regehr, 2010; Castillo et al. 2012).

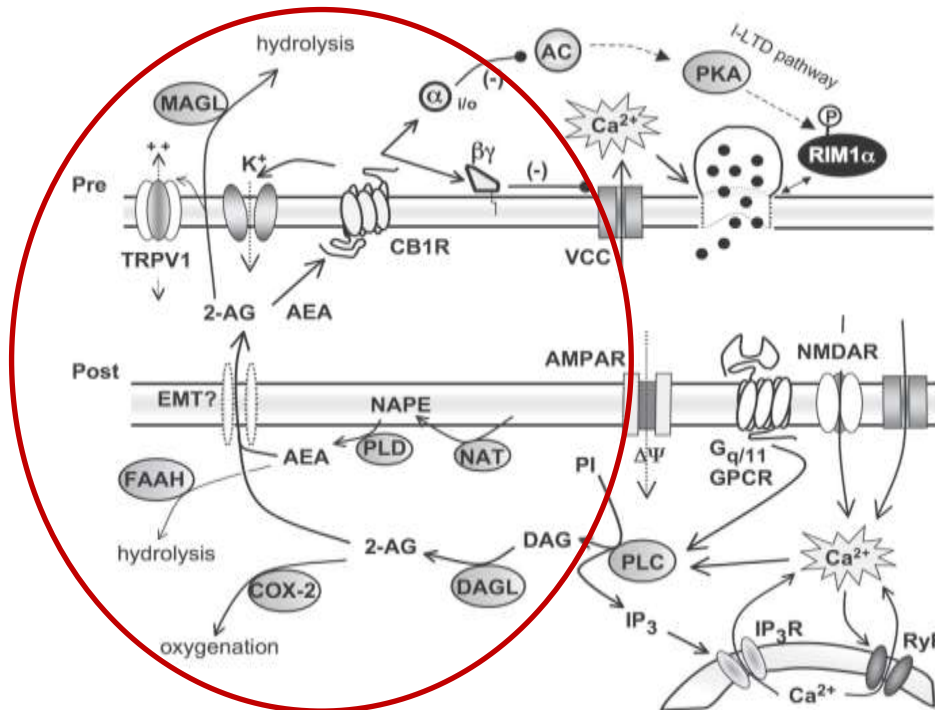

Figure 1. The red circle hightlight the endocannabinoids synthesis/metabolism and their retrograde action in the presynaptic receptors. Additional details are described in the text. Arachidonoylethanolamide or anandamide (AEA), arachidonoylglycerol (2-AG), cyclooxygenase 2 (COX-2), phosphoinositol (PI), diacylglycerol (DAG), diacylglycerol lipase (DAGL), fatty acid amide hydrolase (FAAH), N-acyltransferase (NAT), phospholipase D (PLD), N-arachidonoyl-phosphatidylethanolamine (NAPE), putative endocannabinoid membrane transporter (EMT), monoacylglycerol lipase (MAGL), cannabinoid receptor type 1 (CB_1R) $TRPV_1$ vanilloid receptor (TPRV1R), voltage-gated Ca^{2+} channels (VCC) (Adapted from Gerdeman 2008).

1.2.2. Cannabinoid receptors

The most known cannabinoid receptors are the CB_1Rs and the CB_2Rs, members of the G protein-coupled receptor family (GPCR). Both are primarily coupled to G_i/G_o and their activation leads to inhibition of adenylyl cyclase and voltage-gated calcium channels as well as the activation of potassium channels, mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase (PI_3K)/Akt signalling pathways (Callén et al. 2012).

The CB_1Rs are one of the most abundant GPCRs in the brain, present in corticolimbic areas, hippocampus, basal ganglia, cerebellum, and brain-stem (Irving et al. 2000). They are predominantly present at nerve terminals, exhibiting the highest concentrations in γ-aminobutyric acid (GABA) and glutamatergic neurons (Katona

1999, 2006). Amongst the major neurophysiological effects of CB_1Rs one can point out their important roles in the regulation of network establishment (Harkany et al. 2008) and the modulation of synaptic communication (Freund et al. 2003; Castillo et al. 2012). Besides being mainly neuronal, CB_1Rs can be also found at a lower density in astrocytes and microglia (Duarte et al. 2012; Ramírez et al. 2005). In addition to their classical roles in regulating mood and emotion, they are major players in the modulation of learning and memory (Han et al. 2012; Zanettini et al. 2011). Furthermore, CB_1Rs have also a crucial role in metabolism and thus, they are also localized in peripheral nerves and several peripheral organs related to metabolic homeostasis including adipose tissue, liver, pancreas and skeletal muscle (Mackie 2008; Matias et al. 2008; Silvestri & Di Marzo 2013).

On the other hand, CB_2Rs are mainly found within cells of the immune system, such as macrophages and mast cells, therefore serving as therapeutic targets to control inflammation, pain and immune responses (Maione et al. 2013; Rom & Persidsky 2013). They are also highly expressed in other peripheral tissues, including the spleen, pancreas and in lower density they are also present in the brain, skeletal muscle, liver, intestine and testis, as well as in the adipose tissue (Pertwee 2005; Mackie 2008; André & Gonthier 2010).

Besides the already mentioned functions of CB_1R and CB_2R, they also control cellular functions, such as cell architecture, proliferation, motility, adhesion and apoptosis. Recently, it was found that the two receptors form heteromers in transfected neuronal cells, rat brain pineal gland, nucleus accumbens (NAc) and globus pallidus, which helped to elucidate the mechanism by which CB_2R can negatively modulate CB_1R function (Callén et al. 2012).

Further studies discovered additional sites of action for eCBs and also for synthetic cannabinoid compounds, which support the idea that additional cannabinoid receptors may exist (Köfalvi, 2008). They were named G protein-coupled receptor 55 (GPR55) and G protein-coupled receptor 119 (GPR119) (André & Gonthier 2010). In addition, ion channels are also a possible site of action to eCBs, like in case of transient receptor potential Na^+/Ca^{2+} channel subfamily vanilloid type-1 receptor ($TRPV_1R$) which is activated by anandamide, and also several types of potassium channels, α7 nicotinic receptors and serotonin receptors, among others (Köfalvi, 2008).

1.2.3. Cannabinoid receptors pharmacology

Many ligands are designed based on the structure of Δ^9-THC, which itself is a partial agonists for both the CB_1R and the CB_2R (Bayewitch et al. 1996). Such Δ^9-THC-like ligands are O-2050, a selective, silent (neutral) CB_1R antagonist and JWH133, a CB_2R-selective agonist. Examples for alternative, aminoalkylindol molecules are WIN55212-2, which is a non-selective full agonist at the CB_1R and the CB_2R, and AM630, which is a CB_2R-selective inverse agonist/antagonist. Arachidonyl-2'-chloroethylamide (ACEA) belongs to the thirds larger group, i.e. it is a non-metabolizable anandamide analogue, and a CB_1R and $TRPV_1R$ hybrid agonist. Finally, the fourth large group comprises the diarylpyrazole molecules such as the CB_1R inverse agonist/ GPR55 agonist, antiobesity medicine, rimonabant, or the CB_2R-selective agonists Gp1a (Pertwee 1993, 2005, 2010).

1.3. The pathophysiological roles of the ECS

The ECS is a widespread lipidergic signaling system involved in several physiological functions (Vettor et al. 2008; Tibiriça 2010). Hence, the eCBs play pivotal roles in pathophysiological processes such as obesity, metabolic dysfunctions, stress, anxiety, depression and drug addiction (Urigüen et al. 2004; Gonzalez 2007; Montoya & Vocci 2008; Bhattacharyya & Sendt 2012; Lipina et al. 2012; García-Gutiérrez et al. 2013). Moreover, this system is also implicated in schizophrenia since cannabis consumption has been related to the appearance of psychotic symptoms and schizophrenia (Köfalvi & Fritzsche, 2008; Marco et al. 2011). Thus, cannabinoid receptors are also potential targets to the development of novel treatments for various brain disorders (Izzo et al. 2009; Pertwee 2012).

1.3.1. Metabolic control of ECS

It has been long known that marihuana consumption stimulates appetite (Hollister 1971), decreases body temperature (Borgen et al. 1973) and among others, increases the consumption of highly palatable food, sometimes resulting in significant weight gain (Abel 1975; Berry & Mechoulam 2002). Hence, this complex and pleiotropic endogenous signalling system is a major regulator (both central an peripheral) of

appetite, food intake, energy metabolism and homestasis (Kirkham et al. 2002; Di Marzo & Matias 2005; Matias et al. 2008).

This was proposed based on *in vivo* experiments where rats were injected intraperitoneally with CB_1R and CB_2R agonists. While CB_1R activation retarded the clearance of plasma glucose after the oral administration of glucose, CB_2R agonists exerted the opposite effect. These actions were prevented by the administration of inactive doses of the antagonists of the respective receptors, which, at higher doses, accelerate or retard the clearance of plasma glucose, respectively (Bermudez-Silva et al. 2007).

Moreover, in our lab, recent reports demonstrated that the activation of CB_1Rs inhibit cerebral glucose metabolism in neurons and astrocytes (Duarte et al. 2012), while the genetic ablation rather than the acute pharmacological blockade of CB_1Rs decreases the basal rate of hippocampal glucose uptake in mice (Lemos et al. 2012).

Cannabinoid receptor activation affects energy balance and metabolism through the central control of feeding behaviour and by affecting peripheral metabolism (Després 2007; Matias et al., 2008). Therefore, this fact confers to the ECS a fundamental role in modulating the development of abdominal obesity and associated metabolic abnormalities, which increase the risk of cardiovascular diseases and type 2 diabetes (Engeli et al. 2005; Cote et al. 2007; Lipina et al. 2012). Moreover, it was demonstrated that - depending on the nutritional status - the levels of eCBs oscillate in the limbic forebrain and hypothalamus and inversely proportional to the fed state (Kirkham et al. 2002).

1.4. Cerebral glucose metabolism

Besides the ability of the brain to consume different types of energy substrates, cerebral energy metabolism depends mostly on the availability of glucose provided from the blood flow which is crucial to sustain neuronal activity and function, both in basal and activated states (McCall 2004). Glucose is also an important signal that controls the secretion of hormones by various endocrine cells and activates neurons in the peripheral and CNS (Marty et al. 2007). Brain uses glucose as its major source, being 20-50% of whole body glucose used under resting condition (Fehm et al. 2006;

Matias et al. 2008). This is the most sensitive organ to failure in oxygen and glucose (Abdul-ghani et al. 2007; Matias et al. 2008). Glucose is transported through the endothelial cells of the blood-brain barrier (BBB) and it is used by the brain, mainly in its oxidizable form. Glucose is converted to pyruvate, metabolized in the tricarboxylic acid (TCA) cycle and finally is subjected to oxidation to carbon dioxide and water for full provision of ATP and its high-energy equivalents (McCall 2004). Additionally, glucose can also undergo a non-oxidative metabolism that converts glucose into lactate, which may play a significant role in rapid responses to synaptic activity (Suzuki & Naya 2011). Besides, there is also the storage of glucose as glycogen in astrocytes, representing a considerable resource of glucose energy with high relevance for the hypoglycemia state (McCall 2004).

Therefore, it is expected that a failure in glucose supply or metabolism results in brain dysfunction or even in a permanent damage (Santos et al. 1999; McCall 2004). A prolonged or profound hypoglycemia may affect neurotransmitter metabolism, cerebral blood flow, the BBB and microvascular function, which can lead to coma, seizures and a potentially permanent brain damage (Marty et al. 2007; Matias et al. 2008). Hyperglycemia can also be involved with brain dysfunction (McCall 2004).

1.5. Insulin

Insulin is crucial for the regulation of glucose and lipid metabolism in liver, adipose tissue and muscle, being in charge of the regulation of storage and uptake of digestion products. Besides, this protein is also important in other tissues including brain, pancreas, and vascular endothelium (Siddle 2012). Insulin is synthesized by pancreatic β-cells (van der Heide et al. 2006; Leroith 2002). Although glucose is the key regulator, also fatty acids and amino acids can influence insulin secretion in these cells (Leroith 2002).

Insulin and specific insulin receptors (IRs) are found widely distributed in many peripheral tissues and in CNS networks, related in particular to energy homeostasis (Gerozissis 2004). In the brain, IRs are expressed in the highest concentrations in olfactory bulb, hypothalamus, cerebral cortex, cerebellum, hippocampus (Havrankova & Roth, 1978) and also in the pituitary intermediate lobe (Unger & Betz 1998).

The IR is a membrane-bound tyrosine kinase receptor which are tetramers composed of two extracellular α-subunits and two transmembrane β-subunits (Taha & Klip 1999; White 2003). In the CNS, they differ from their peripheral counterparts by having lower molecular weights of both α- and β-subunits (White 2003).

Extracellular insulin binds to its receptor inducing the autophosphorylation of tyrosine residues of the β-subunit followed by tyrosine phosphorylation of the insulin receptor substrates (IRS) responsible to activate downstream pathways like the phosphatidylinositol-3-kinase (PI_3K/Akt) and MAPK/ERK pathways (Taha & Klip 1999; White 2003; van der Heide et al. 2006). The PI_3K/Akt pathway leads to Akt activation, which induces glucose transporter translocation to the plasma membrane increasing glucose uptake (Benomar et al. 2006).

In spite of the differences between the characteristics of IRs in neurons and the periphery, mechanisms that regulate the effects of insulin in the brain show similarities with peripheral insulin action (Gerozissis 2004). The PI3K–PKB/Akt signalling pathway proved to be an important component involved in insulin-induced neuroprotection (van der Heide et al. 2006) and several lines of evidence suggest an important role of PKB/Akt in regulating the effects of PI_3K in neuronal survival (Rodgers & Theibert 2002).

The regulation of food intake through insulin signalling does not occur only through peripheral processes but also has central components (Gerozissis 2004). The circulating brain insulin is mainly derived from peripheral insulin (pancreatic origin) since it crosses the BBB, depending on its levels in plasma, by a saturable mechanism. This transport provides a mechanism by which peripheral insulin can act within the CNS as a regulatory peptide (Banks 2004; Plum et al. 2006) and thus being involved, for example, in the feedback loop between brain peptides and food intake (Gerozissis 2004). Insulin's actions are influenced and dependent on the effect of other hormones and peptides such as leptin, corticosteroids, neuropeptide Y (NPY) and galanin which are also implicated in energy homeostasis (Gerozissis 2004; Matias et al. 2008). Moreover, insulin also regulates mechanisms that include learning, memory, neuronal survival and reproductive endocrinology (Gerozissis 2004). Hence, an impairment of insulin availability/signalling, such a failure in PI_3K–PKB/Akt pathway, both in

peripheral tissues or in the brain, can lead to serious metabolic or endocrine pathologies, such as diabetes, obesity and mental or reproductive disorders (Gerozissis et al. 2001; Gerozissis 2004; White 2003).

Similarly to insulin, insulin-like growth factor (IGF) is abundant in the CNS (Matias et al. 2008; Johansson et al. 2013; Bondy & Cheng 2004) and its signalling also regulates metabolic functions in the brain (Broughton & Partridge 2009; de la Monte 2012). There are pieces of evidence of a cross talk between IGF and insulin and their respective receptors and thus, in high, non-physiological concentrations IGFs are capable of IR activation and insulin can activate the insulin-like growth factor type-1 receptor (IGF1R) (Denley et al. 2007). Moreover, it was previously found that IR and IGF1R can form homo- and heterodimers (Slaaby et al. 2006; Siddle 2012).

Although IGF1R plays a crucial role in the facilitation of glucose uptake in the neuronal processes in the early postnatal brain, IGF1 largely disappears from the adult brain (Cheng et al. 2000). Insulin reaches cerebral levels in the adult brain 10-100-times higher than in the plasma which is the range required to activate the 10-100-times less insulin-sensitive hybrid IGF-1R/IR heterodimer (Slaaby et al. 2006). Furthermore, it has been shown that IGF1 and insulin (500 nM) both can trigger long-term depression at the Schäffer-collaterals in CA1 synapses, but insulin's action can be prevented only by an IR antibody in the perfusion medium, while IGF1-LTD was sensitive exclusively to an IGF1R antisera (Huang et al. 2004). Altogether, it is less likely but not fully excluded that IGF1R may mediate some of insulin’s action in the adult brain.

1.6. Diseases of impaired insulin signalling

1.6.1. Diabetes type 1, 2 and 3

The most common forms of diabetes are the type 1 and the type 2. Type 1 diabetes is an autoimmune disease associated with genetic susceptibility and pancreatic β-cell death. Several studies described the major histocompatibility complex (MHC) as the main genetic determinant of this type of diabetes, which is characterized by the total lack of insulin production as a consequence of progressive failure and death of pancreatic β-cell. On the other hand, the predominant cause of type 2 diabetes, which

represent approximately 90% of diabetic cases is related to lifestyle factors, namely diet, a sedentary life, stress, smoking and obesity. Nevertheless, type 2 diabetes is also associated with genetic predisposition or related pathologic factors, such as hypertension (Negre-Salvayre et al. 2009).

Both type of diabetes are usually associated with long-term macrovascular and microvascular complications, which have repercussions in different organs and tissues. The most common macrovascular lesion is atherosclerosis in blood vessels. However, the most abundant complications are microvascular, which are related with hyperglycemia, such as nephropathy, retinopathy, and peripheral neuropathy (Malecki 2004; Brownlee 2005; Negre-Salvayre et al. 2009).

Type 2 diabetes is characterized by peripheral and hepatic insulin resistance, in addition to a progressive pancreatic β-cell death in the islets of Langerhans (Boura-Halfon & Zick 2009; Kawahito 2009). This means the inability of insulin to increase glucose uptake in the peripheral tissues and repress gluconeogenesis in the liver, which is due to the incapacity of cells to respond to insulin's actions (Regazzetti et al. 2009; Boura-Halfon & Zick 2009). Consequently, β-cell function is increased, resulting in hyperinsulinemia (high levels of insulin circulating in the blood) leading to even greater insulin resistance, tissue stress, ROS production and β-cell death – with the consequent onset of type-1 diabetes (Lipina et al. 2012; Newsholme et al. 2007; Friedrich 2012; Kawahito 2009; Brownlee 2005). All in all, the consequence of either deficient insulin secretion or insulin resistance is hyperglycemia in the blood, and energy deprivation of the peripheral cells (Brownlee 2005).

Since at least the 1920's it is known that disturbances in glucose and insulin metabolism in diabetes can affect the CNS. In 1922, the first pieces of evidence of brain dysfunction induced by diabetes was reported (Miles & Root 1922). The term "diabetic encephalopathy" was introduced to describe this condition (DeJong 1950) in which there is a gradually developing end-organ damage to the CNS associated with diabetes progression (Brands et al. 2003).

Furthermore, several studies suggested that disturbances in cerebral insulin signalling and in glucose homeostasis could be the cause of neurodegenerative disorders like Parkinson's and Alzheimer's diseases (Blum-Degen et al. 1995; Steen et al. 2005; Craft 2009; Kaidanovich-Beilin et al. 2012). It has been demonstrated that

experimental brain diabetes produced by intracerebral administration of streptozotocin (STZ) leads to cognitive impairment and dysfunction on acetylcholine homeostasis, features of AD (Lester-Coll et al. 2006). Hence, this disturbances of glucose homeostasis and cerebral insulin resistance (Correia et al. 2012) suggested that AD may represent a neuroendocrine disorder and the term “type 3 diabetes” was proposed (Steen et al. 2005).

1.6.2. Glucocorticoid-induced insulin resistance

Glucocorticoids (corticosterone in the rodents and cortisol in man) are hormones produced in the adrenal cortex as a physiological response to stress, under the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Burén et al. 2002; Hill & McEwen 2010; Dalmazi et al. 2012). These hormones are involved not only in stress response but also in the homeostasis of energy metabolism, immune and inflammatory responses (Sarabdjitsingh et al. 2010). Thus, glucocorticoids trigger rapid and adaptive responses to stress, promoting glucose mobilization and redirecting energy stores (Hill & McEwen 2010). These receptors include glucocorticoid and mineralocorticoid receptors, members of the nuclear receptor family [MR and GR] (Funder 1997).

Previous studies suggested an action of glucocorticoids in the central regulation of appetite, both directly and indirectly. Thus, a hypersensitivity to these hormones or an increase of its circulating levels (even in therapeutic doses), are involved with the induction of food intake and the development and maintenance of obesity syndromes (Tataranni et al. 1996; Zakrzewska et al. 1999).

Hence, the chronic exposure to glucocorticoid excess, for example of cortisol, can result in a number of chronic metabolic complications and thus, in the clinical condition of endogenous hypercortisolism (EH), the Cushing’s syndrome/disease in which frequently associates with glucose intolerance (Schneiter & Tappy 1998). This is due to the glucocorticoid-induced global insulin resistance which can be reversible upon cessation of the glucocorticoid administration (Amatruda et al. 1985; Sonino et al. 1998; Lansang & Hustak 2011). Hypercortisolism can also increase in the rate of incidence in diabetes and sporadic Alzheimer's disease (AD) likely due to cerebral

insulin resistance (Solas et al. 2013). Diabetes is an important contributing factor to the morbidity and mortality of the patients with EH (Dalmazi et al. 2012; Yi et al. 2012; Ferris & Kahn 2012). Moreover, chronic stress and hyperglucocorticoidism in animal models permanently alter the activity and the metabolism in the dopaminergic areas, including the NAc, which may signify stress-induced impairment in the reward area (Bock et al. 2012; Barik et al. 2013).

1.7. Cannabinoid receptors and insulin

Peripheral CB_1R can influence insulin action in several tissues including the adipose tissue, the liver and the skeletal muscle, independently of central activation of CB_1R or food intake (Matias et al. 2008; Nogueiras et al. 2009). This occurs through the insulin-stimulated Akt phosphorylation by eCBs which leads to the decrease of glucose uptake in skeletal muscle cells. Besides, CB_1R blockade increases the insulin sensitivity of the skeletal muscle (Eckardt et al. 2009) which is dependent of the activation of PI_3K (Esposito et al. 2008). Hence, the activation or inhibition of this receptor can lead to either enhancement or attenuation of insulin-mediated signalling in skeletal muscle (Lindborg et al. 2010; Lipina et al. 2012). Furthermore, in diabetes mouse models, CB_1R activation prevented the autophosphorylation of IRs and the activation of downstream signals in pancreatic β-cells as well as in non-insulin-secreting cells (Kim et al. 2011).

Recently, it was found that CB_1Rs can form a heteromeric complex with receptors tyrosine kinase (RTKs) (Berghuis et al. 2007; Dalton & Howlett 2012) including the IRs (Kim et al. 2012). Thus, the kinase activity of the receptor is inhibited which reduces the Akt-mediated phosphorylation of the proapoptotic protein Bad, leading to β-cell death. This study has provided direct evidence of physical and functional interactions between CB_1R and IR, suggesting a mechanism by which the peripherally acting CB_1R antagonists improve insulin action in insulin-sensitive tissues independent of the other metabolic effects of CB_1Rs (Kim et al. 2012).

The activation of CB_2Rs is also involved in insulin secretion of pancreatic β-cells by regulating intracellular calcium signals that leads to the decrease of insulin secretion (Juan-Picó et al. 2006).

1.8. The reward system and insulin

The reward system was identified as the behavior mediator motivated by pleasure, evoked by food, drugs of abuse or sex (Wise & Rompre 1989; Bruijnzeel et al. 2011). Thereby, reward system drives behaviors to those usually associated with positive outcomes (Kelley & Berridge 2002). A dysregulation in the brain reward circuitry can lead not only to drug addiction but also promote continued overfeeding behavior - the “non-homeostatic feeding”, which is controlled by the mesolimbic pathway (Zheng et al. 2009; Wang et al. 2010). Thus, the high consumption of palatable foods can be a result of a variety of sensory stimuli and emotional states or feelings, and provokes neuroadaptative changes in this brain area (Berthoud et al. 2011). Thus, this induced overfeeding behaviour can contribute to the human overweight prevalence increase and ultimately to obesity (Wang et al. 2010; Berthoud et al. 2011; Davis et al. 2010).

One of the most important anatomical substrate areas involved in the reward and motivation circuitry is the NAc (also called ventral striatum in primates and men) which receives dopaminergic inputs from the ventral tegmental area (VTA) of the midbrain. A dysfunction on this brain reward area may contribute to anhedonia, decreased sex drive, social withdrawal, and other symptoms of depression (Nestler & Carlezon 2006; Wang et al. 2010).

The postprandial reward effect is the result of accumbal activation induced by dopaminergic inputs from the VTA of the midbrain and it regulates both normal feeding behavior and aversive motivational processes (Kelley 2004; Salamone & Correa 2012). It was demonstrated that a decrease in lever pressing for food reward was associated with a reduction on dopamine (DA) turnover in this brain reward area (Davis et al. 2008; Berthoud et al. 2011), which is in concert with dopamine's crucial role in the regulation of feeding, motivation and pleasure (Palmiter 2007). These alterations in reward behaviors and in the mesolimbic dopamine signaling can be an outcome of both high-fat diet and obesity *per se* (Berthoud et al. 2011). Hence, some recent studies proved that reward and metabolism are probably regulated by an overlapping brain circuitry (Davis et al. 2010). Central resistance to insulin and leptin can impair the control of systemic energy homeostasis in brain reward areas apart from the hypothalamic system (Berthoud et al. 2011). Hence, this raises the possibility that insulin and leptin may regulate reward-related behavior through providing

feedback on the mesolimbic circuitry (Speed et al. 2011). Lines of evidence support the notion that alterations in insulin and glucose can influence mesoaccumbal DA release (Bello & Hajnal 2008). A recent study demonstrated that the insulin-induced long-term depression (LTD) of mouse excitatory synapses onto VTA dopamine neurons employs the presynaptic CB_1R-mediated inhibition of glutamate release (Labouèbe et al. 2013).

Chapter 2. Objectives

The aim of this study was to map and characterize the multifaceted role of the ECS in the regulation of cerebral glucose. Based on the involvement of this system in the physiological control of appetite and satiety, mainly through CB_1R action, we sought to investigate whether insulin affects (increases) glucose uptake in the NAc and if this purported action of insulin is dependent on local CB_1R signalling.

NAc is involved in postprandial satiety elicited in part by insulin and the consequent termination of food-seeking behaviour. Moreover, glucocorticoid excess can lead to insulin resistance, diabetes or Alzheimer's disease, and recent findings suggested that glucocorticoids can trigger endocannabinoid release. Thus, we aimed at understanding how the glucocorticoid dexamethasone affect insulin-mediated glucose uptake in the nucleus accumbens and whether this is dependent on local CB_1R signaling.

Since brain glucose hypometabolism is a preclinical symptom of Alzheimer's disease and CB_2Rs are upregulated in AD patients and in animal models of AD, we also aimed at study the putative regulation of glucose uptake by CB_2R in the hippocampus of healthy rodent brain and under Aβ burden.

Chapter 3. Materials and Methods

3.1. Animals

All studies were conducted with the principles and procedures outlined in the European Union (EU) guidelines (86/609/EEC) and by FELASA, in accordance with the recommendations of the NC3Rs Reporting Guidelines Working Group (2010), and were approved by the Portuguese Ministries of Agriculture, the local Animal Care Committee of the institutes (license numbers: 280279-31-A and 025781 respectively) and the Federation of Laboratory Animal Science Associations. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort. Animals were housed in the specific pathogen-free facilities, with 12 h light on/off cycles, under controlled temperature (23±2 ºC), and *ad libitum* access to food and water.

For *in vitro* experiments, six-week-old male Wistar rats and male mice of the CD-1 strain were purchased form Charles-Rivers (Barcelona, Spain). Middle-aged (12 months) C57Bl/6j mice containing the human transgene, APP695 with the double mutations at KM670/671NL transgenic line 2576, (hereafter, TgAPP) mice, expressing Aβ-burden but no evident cell loss (Hsiao et al. 1996) and the age-matched C57Bl/6j (hereafter referred as WT [wild-type]) mice were genotyped at and provided by the Cajal Institute, Madrid, Spain. At 12 months, TgAPP mice did not differ in weight (WT: 39.6±2.4 g; TgAPP: 38.6±2.3 g, n=5 randomly chosen mice) and did not show brain amyloidogenic plaques, but had compromised new object recognition compared to WT mice.

Soluble Aβ 1-42 peptide (2 nmol) or vehicle (0.1% NH_3) was injected in young male Wistar rats to induce AD-like pathology. This leads to an accumulation of soluble but not aggregated forms of Aβ in the hippocampus, causing delayed memory impairment without evident acute effects (Canas et al. 2009). Eighteen days after Aβ or vehicle injection and 1 day after memory tests - all carried out by colleagues - rats were sacrificed on the day 18 post-injection for *in vitro* glucose uptake experiments in brain slices.

3.1.1. Experimental model of diabetes

Experimental model of type-1 (insulinopenic) diabetes was induced in Wistar rats with 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose (streptozotocin or STZ) (Calbiochem, Merck Biosciences, Germany). STZ solution was prepared freshly at the concentration of 60 mg/mL in citrate buffer (pH 4,5). Adult rats after four hours of food deprivation were injected intraperitoneally with a dose of 60 mg/kg of the body weight. Body weight and glycemia (blood glucose levels) were determined before the injection and three and fifteen days post-injection. Glycemia was measured from the tail vein through the glucose oxydase method, using a glucometer and reactive test stripes (Elite-Bayer SA, Portugal).

STZ induces diabetes within 3 days by destroying the β cells (Akbarzadeh et al. 2007). Three days after injection, glycemia was determined to confirm rats' diabetic condition: Animals were considered diabetic only if exhibiting glycemia > 300 mg/dl (Table 1). Animals were housed in metabolic cages (two animals for each cage) with two bottles of water under feeding and metabolism control until the scarification day (15 days post-injection) for glucose uptake experiments. Body weight was determined again before sacrifice (Table 1).

Table 1. Body weight and glycemia of control and STZ-injected Wistar rats (n=9).

	Weight (g)		**Glycemia (mg/dl)**	
	Control	**STZ-treated**	**Control**	**STZ-treated**
Before treatment	n.d.	262.4 ± 1.9	106.7 ± 10.4	113.6 ± 0.7
3 days after treatment	n.d.	n.d.	n.d.	477.3 ± 10.1
15 days after treatment	369.4 ± 9.9	251.5 ± 2.0	106.1 ± 5.9	505.4 ± 19.8

n.d.: not determined.

3.1.2. Fasted rats

Male Wistar rats used for glucose uptake experiments were subjected to food privation for 16 hours before sacrifice. Body weight was determined before and after the fasting period. Fasted rats lost 6.6 ±0.21 % of their body weight.

3.2. *In vitro* glucose uptake assays

Around 14:00 o'clock each experimental day to reduce putative circadian hormonal effects, animals were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine.

A previously optimized *in vitro* glucose uptake protocol for acute brain slices was used (Lemos et al. 2012). This protocol allows the simultaneously comparison the effect of various treatments in pairwise arrangement.

Brains were rapidly removed and placed in ice-cold carboxygenated (95% O_2 and 5% CO_2) Krebs'- HEPES (KH) solution with the following composition (in mM): 133 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 5.5 glucose, and 10 HEPES (pH 7.4). Nuclei accumbens (rat brain) or hippocampi (mice/rat) were dissected on ice within 4 min after decapitation, and sliced into 450 µm- thick transversal slices with the help of a McIlwain tissue chopper (Ted Pella, CA, USA). Whenever possible, we used the same rats' hippocampal and accumbal slices for the two different studies to economize the number of animals used.

From each animal and for each of the experimental conditions (in different chambers), five hippocampal and/or 3 accumbal slices were used from the pair of NAc/hippocampi. The slices were transferred into a multichamber slice incubator and incubated in 50 mL of continuously gassed (95% O_2 and 5% CO_2) KH solution at 37 ºC until the end of the experiment.

Acute slices were first subjected to 60 min of preincubation necessary for metabolic recovery (see our previous study: Lemos et al. 2012). Subsequently, drugs or their vehicle dimethyl sulfoxide (DMSO) (0.1% v/v) were bath applied, and 5 min later, the following radioactive glucose analog was added for a period of 30 min: 2-^{3}H(N)-deoxy-D-glucose (^{3}H-2-deoxyglucose; ^{3}HDG; 2.5 nM; 60 Ci/mmol; American Radiolabeled Chemicals - ARC). The length of this incubation period was chosen based on preliminary results. Upon completing the incubation, the slices were washed gently but extensively (twice) in Petri-dishes with ice-cold uptake solution and collected in 1 mL NaOH (0.5 M). Slices were left to dissolve overnight then boiled at 90ºC for 20 min to allow homogenous disintegration for protein and radioactivity measurements.

Of each sample, 800 µL were collected in scintillation vials containing 2.5 mL of scintillation liquid (Zinsser Analytic, Germany) and assayed for ^{3}H (value X disintegration/minute [dpm]) counts with the help of a Tricarb β-counter (dual-label protocol) (PerkinElmer, USA) The rest of the sample was used for the quantification of total protein (P mg) with the bicinchoninic acid assay (see below).

The incubation bath (181.8 µL) containing the radioactive glucose analogs was also sampled and assayed for ^{3}H dpm (value A). Values A represent 1 µmol quantity of cold glucose molecules, because in a 5.5 mM glucose solution, a 181.8-µL volume contains 1 µmol glucose. Knowing how much ^{3}H signal is associated with 1 µmol glucose in the initial assay medium allows then determining how many nmols of glucose were taken up by the slices: The accumulation of the hardly metabolizable glucose analogue ^{3}HDG (Figure 2) in the slice represents the total uptake which shows linearity in the 30 min period. Hence, if A = 1000 nmol (1 µmol), the total uptake was X/A nmol/mg protein in the slice.

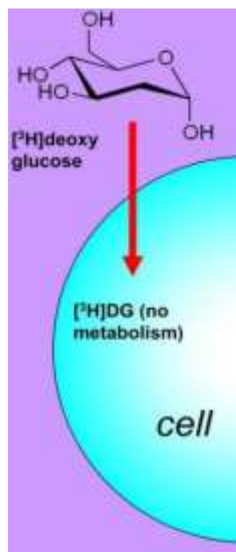

Figure 2. Schematic representation of the cell uptake of the radioactive glucose analog, the non-metabolizable ^{3}H-2-deoxyglucose.

3.2.1. Protein quantification by the bicinchoninic acid method

Quantification of protein was carried out using the bicinchoninic acid (BCA) assay (Merck Biosciences, Germany), a colorimetric method. A standard curve with bovine serum albumin (BSA) (Sigma-Aldrich, Portugal) was prepared in NaOH, using the

following concentrations: 0; 0.25; 0.50; 1; 2; 3; 4 and 5 mg/ mL of BSA. To prepare the standard curve, 50 µL of each concentration of BSA are added, in duplicate, in a 96 multi-well plate/dish. The same amount is added for protein samples, also in duplicate.

The working reagent was created by mixing 50 parts of reagent A (BCA reagent) with 1 part of reagent B- copper (II) sulfate solution (BCA reagent). Then, 200 µL of BCA working reagent was added to all samples and the dish was placed in a 37ºC incubator for 1 hour. The absorbance was measured at 562 nm in a spectrophotometer. Sample concentrations are estimated using the BSA standard curve.

3.3. Fluorescent glucose uptake assays in mice coronal brain slices

Around 14:00 o'clock each experimental day to reduce putative circadian hormonal effects, young male C57bl/6j mice were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine. Brains were quickly removed into the ice-cold carboxygenated KH solution (composition described above) and mounted on metal platforms to cut 300 µm-thick coronal slices with the help of a vibratome. Slices were placed in the carboxygenated KH solution at room temperature (RT) for 60 min.

Hemisphere slices were mounted on a coverslip placed on a RC-20 superfusion chamber in a PH3 platform (Warner Instruments, Harvard, UK) on the stage of an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss, Germany). We placed a cover glass above which helps smoothen liquid flow. This helps to prevent optical noise owing to fluctuation of the medium surface level. The experiment was conducted with a continuous superfusion system, at a rate of 0.5 mL/min in a closed circuit, with a carboxygenated KH solution at RT.

Subsequently, acquired images of a defined region of hippocampus in the hemisphere coronal slices were captured with CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA) at every 30 seconds during a total of 30 minutes, using a 5× PlanNeofluar-objective (NA 0.25, inverted Axiovert 200M fluorescence microscope, Carl Zeiss, Germany, coupled to a Lambda DG-4 integrated 175 Watt light source and wavelength switching excitation system [Sutter Instrument Company, Novato, CA, USA] allowing real-time video imaging) and band-pass filters for excitation (BP470/40)

and emission (BP525/50), with identical parameters throughout the study. The average value of pixel intensities was evaluated at each time point. Values were processed using the MetaFluor software (Universal Imaging Corporation, Buckinghamshire, UK).

Basal ratio was measured during the first minute of the experiments, which allowed the evaluation of autofluorescence (AF) (recording of 4 images to set zero level). After the first minute, the fluorescent glucose analogue tracer: 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; 30 µM) was bath applied in the reservoir with the carboxygenated KH solution. This allows the real-time monitorization of 2-NBDG uptake, useful in the analysis of mechanisms underlying glucose uptake and concomitant cellular functions in mammalian cells (Yamada et al. 2000; Yamada et al. 2007). After 15 minutes of basal line we bath applied JWH133 (1 µM), or GP1a (100 nM) or their vehicle, DMSO (0.1% v/v). Each condition was carried out in duplicate per animal. The experiments were carried as exemplified in the scheme bellow (Figure 3).

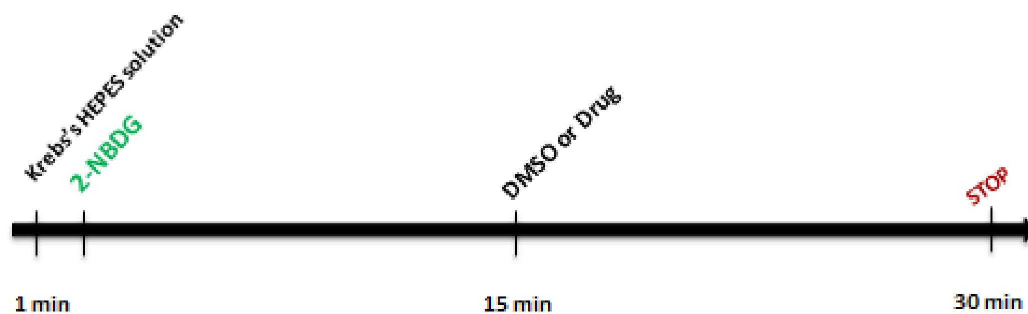

Figure 3. Schematic representation explaining the timeline of the experiments.

To data processing, AF is subtracted to the raw data (Figure 4A) collected with the help of MetaFluor software. The initial rapid increase in 2-NBDG intensity is followed by a transient dip which has been previously published by O'Neil and colleagues (2005). Excluding the initial phase from the curve fitting, data points which represent individual intensity values from the whole hippocampal slice, can be simulated with the following curve:

$$\mathbf{Y = T_{max} \times X / (K_d + X) + M \times X + AF,}$$

where Y stands for the intensity (arbitrary unit), X is the time, T_{max} represents the maximum number of transporters for 2-NBDG, K_d is the inverse of the affinity of 2-

NBDG to its transporters, while M stands for the constant for the "metabolic drain" (2-NBDG is a metabolizable glucose analog) which we assume to be steady for the sake of simplicity, whereas AF is the value to be subtracted from the raw data resulting in the plots in Figure 4B.

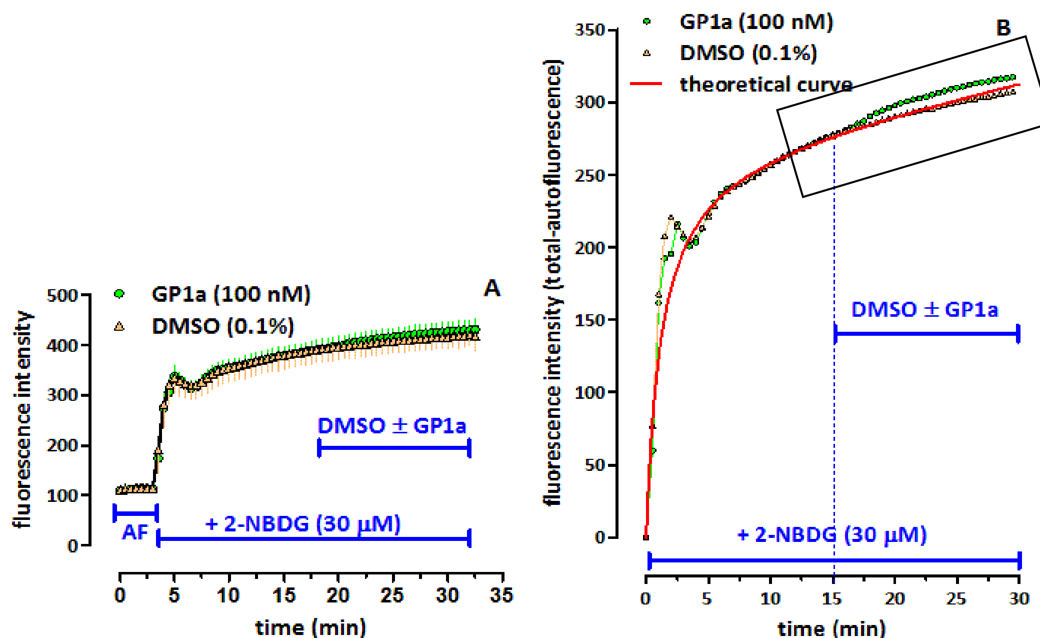

Figure 4. Representative graph of the real-time monotorization of fluorescence intensity changes. Auto-fluorescence (AF) was subtracted to raw data processing. The theoretical curve (red line) is represented as an illustration, calculated for the average of the GP1a curves. Points represent the mean ± SEM of 5 independent experiments (animals) performed in duplicate.

Approx. seven minutes after the addition of 2-NBDG, the rate of increase in fluorescence intensity turns fairly linear, thus allowing recording a ~8 min pretreatment period and an additional 15 min post-treatment phase, which altogether ~23 min period is marked with the rectangle. This represents the theoretical curve which is presented as the Y=0. Points represent the mean ± SEM of 5 independent experiments (animals) performed in duplicate.

3.4. Data presentation and statistical analysis

All data are expressed as means ± SEM of the indicated number of independent observations (n≥5). Raw metabolism and normalized uptake data were tested for normality by the Kolmogorov–Smirnov normality tests. If data suggested Gaussian

distribution, statistical significance was calculated by one sample *t*-test. In case of glucose uptake, the control equals 100%. If more than two groups were compared, one-way ANOVA with Bonferroni's *post-hoc* test was performed. Data from paired experiments were compared with the pairwise version of student's *t*-test or ANOVA, and a value of $p<0.05$ was accepted as a significant difference.

For the fluorescent glucose uptake assay, normalized data was tested for normality by the Kolmogorov-Smirnov normality tests and statistical significance was calculated by one-sample *t*-test against a hypothetical control value and a value of $p<0.05$ was accepted as a significant difference. Groups were compared with the DMSO group with the help of Repeated Measures ANOVA followed by Dunnett's Multiple Comparison Test (*$p<0.05$, **$p<0.01$).

3.5. Chemicals

HEPES, DMSO, $CaCl_2$, $MgSO_4$, $NaHCO_3$ and KH_2PO_4 were purchased from Sigma–Aldrich Portugal (Sintra, Portugal). NaCl, KCl, NaOH, HEPES and D-glucose were purchased from Calbiochem, Merck Biosciences, Germany. 2-NBDG was purchased from Invitrogen (Carslbad, California, USA). Non-water soluble substances and 2-NBDG were dissolved or reconstituted in DMSO, and stored aliquoted at -20ºC. The synthetic glucocorticoid dexamethasone 21-phosphate was purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in 0,9% saline, and then diluted into vehicle (saline or 0,2% DMSO in saline]. Insulin, O-2050, dexamethasone (DEX), THL, JZL184, WWL70, mifepristone, OMe-Tyr, AM630, DuP697, JWH133, GP1a, LY2183240 and WIN55212-2 were obtained from Tocris Bioscience (Bristol, U.K.), except SR141716A which was bought from ARC.

Chapter 4. Results

4.1. CB_1R mediation of insulin-induced glucose uptake in the NAc of wild-type rats

4.1.1. Insulin and CB_1R blockade increased accumbal glucose uptake

NAc metabolism was measured through an *in vitro* procedure which allows glucose uptake quantification as already described (Lemos et al. 2012). Briefly, glucose uptake was measured through the uptake of non-metabolizable ^{3}H-2-deoxyglucose by the acute slices. Control was normalized to 100%, representing the glucose uptake without any treatment. From the two concentrations tested, insulin at 30 nM had no effect (see Figure 9 below). However, at 300 nM, insulin increased glucose uptake in the accumbal slices (n=4, $p<0.05$), thus this concentration was selected for subsequent experiments (Figure 5).

The blockade of the CB_1R by the selective neutral antagonist, O-2050 (500 nM) significantly increased glucose uptake in accumbal slices (n=16, $p<0.05$) similarly to insulin. Moreover, when both were present, mutual occlusion was observed between the two treatments (n=14, $p<0.05$). CB_1R activation by WIN55212-2 (WIN, 500 nM) had no significant effect on basal and insulin-stimulated glucose uptake (Figure 5).

Next, we tested the dependence of insulin's action on IGF1Rs. Fifteen minutes of preincubation by the IGF1R antagonist, I-Ome-Tyrphostin AG (OMe-Tyr, 1 μM) failed to affect either accumbal glucose uptake per se and or the insulin-induced glucose uptake (n=8, $p<0.05$) (Figure 5), indicating the sole involvement of the insulin receptors in the observed effects.

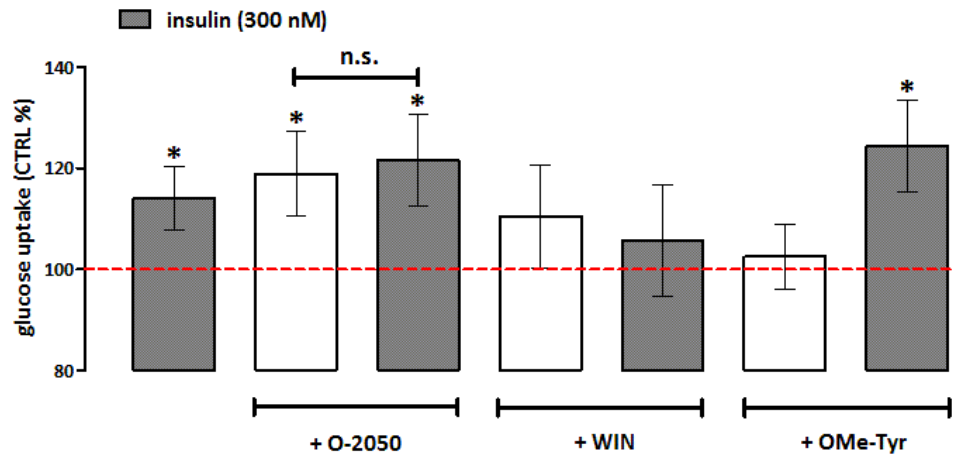

Figure 5. The pharmacology of insulin effect on glucose uptake, in 450 µm-thick accumbal slices of male Wistar rats, under the blockade (O-2050, 500 nM) or activation (WIN, 500 nM) of CB_1R as well as the blockade of IGF1R (OMe-Tyr, 1 µM). Data represent the mean ± SEM of n≥6 and statistically significant differences on glucose uptake were calculated by the one sample *t*-test against the hypothetical value of 100 (* $p<0.05$, relative to control [100%]; n.s., not significant (between O-2050 vs. O-2050+insulin).

4.1.2. Dexamethasone impairs insulin's action - an effect reversed by CB_1R blockade

The glucocorticoid dexamethasone (DEX, 10 µM) prevented insulin from increasing glucose uptake in accumbal slices. Notably, the facilitator effect of the CB_1R agonist, O-2050 persisted in the presence of DEX, and as before, insulin failed to modulate the glucose uptake any further under this condition (n=8, $p<0.01$; $p<0.05$) (Figure 6).

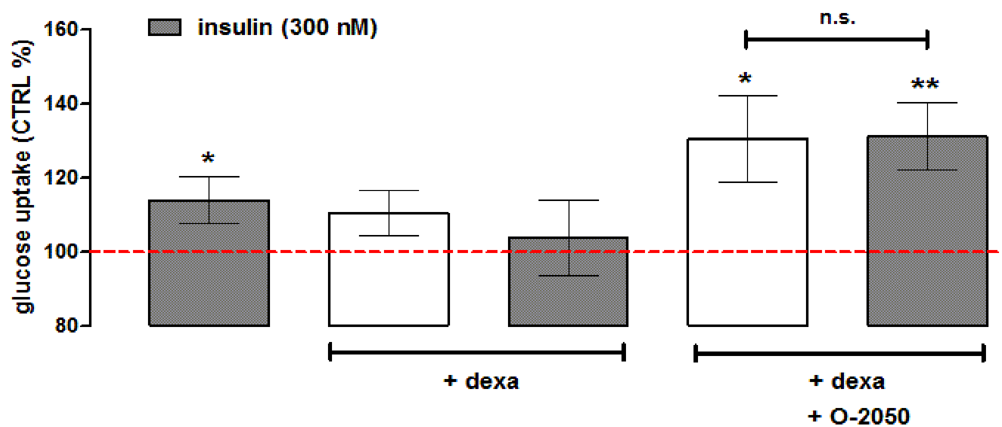

Figure 6. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices of male Wistar rats, in the presence of dexamethasone (DEX, 10 µM) alone or in combination with the CB_1R antagonist, O-2050 (500 nM). Data represent the mean ± SEM of n≥6 and statistically significant differences on glucose uptake were calculated by the one sample t-test against the hypothetical value of 100 (* p<0.05, ** p<0.01, relative to control [100%]; n.s., not significant (between DEX+O-2050 vs. DEX+O-2050+insulin).

4.1.3. The inhibition of endocannabinoid synthesis or metabolism affects insulin's action on accumbal glucose uptake

We sought to investigate insulin-evoked cerebral glucose uptake in the presence of endocannabinoid synthesis or metabolism inhibitors. To this end, we incubated the slices for one hour during the recovery period with the DAGL inhibitor, tetrahydrolipstatin (THL, 10 µM). THL exhibited a tendency to increase glucose uptake such to a similar level as O-2050 did. When DAGLα-mediated 2-AG synthesis was blocked by (THL, 10 µM) no effects were produced, alone and when combined with DEX (10 µM). However, THL prevented the inhibitory action of DEX on the insulin-mediated glucose uptake (n=9, p<0.05). On the other hand, inhibitors of the 2-AG metabolizing enzymes MAGL (by JZL184, 1 µM) and the α/β-hydrolase domain 6 (by WWL70, 1 µM), prevented insulin-induced glucose uptake in NAc (Figure 7).

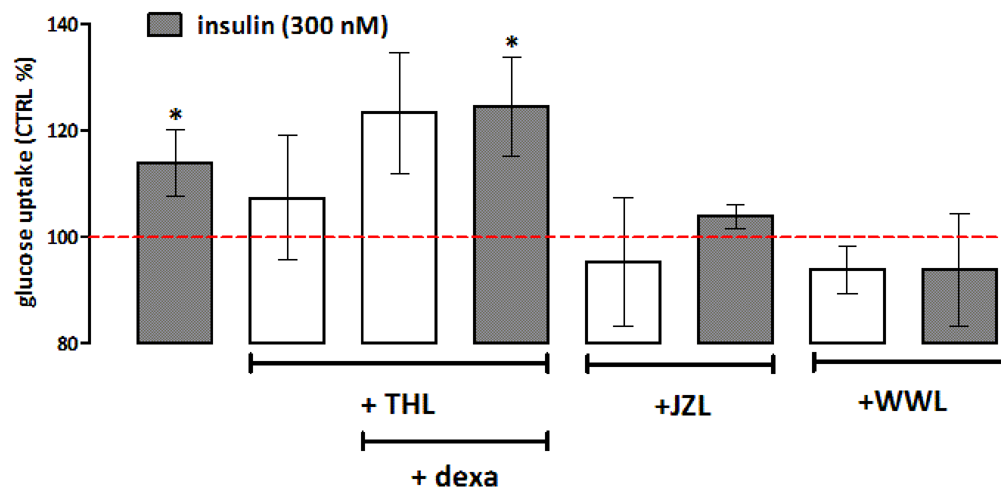

Figure 7. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices of male Wistar rats, in the presence of inhibitors of 2-AG synthesis: THL (10 µM) or metabolism: JZL184 (1 µM) and WWL70 (1 µM). Data represent the mean ± SEM of n≥6 and statistically significant differences on glucose uptake were calculate by the one sample *t*-test against the hypothetical value of 100 (* p<0.05, relative to control).

4.1.4. Insulin effects in glucose uptake in acute accumbal slices from fed, fasted and STZ-induced diabetic rats

Insulin at the concentration of 300 but not at 30 nM, significantly increased glucose uptake in NAc slices (n=14, p<0.05). On the other hand, insulin's effect was reverted by 16 hours fasting, that is, insulin (300 nM) significantly inhibited the basal glucose uptake (n=6, p<0.01). Finally, in accumbal slices of STZ-induced diabetic rats, insulin failed to produce any effect on glucose uptake (Figure 8).

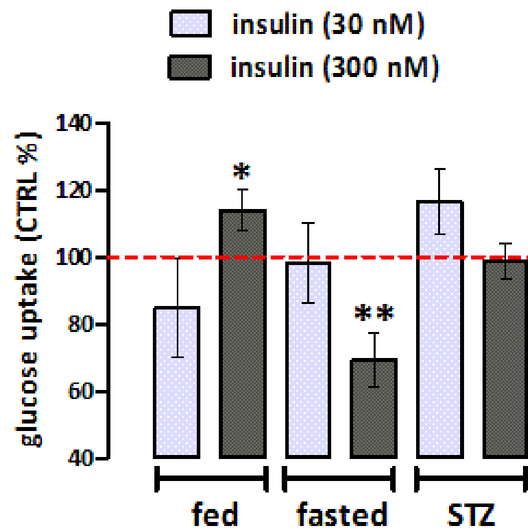

Figure 8. The pharmacology of insulin effect on glucose uptake in 450 µm-thick accumbal slices. Two concentrations of insulin were tested (30 nM and 300 nM) in fed, fasted and STZ-injected diabetic rats. Experiments were carried out in pairwise arrangement. Data represent the mean ± SEM of n≥6 and statistically significant differences on glucose uptake were calculated by the one sample *t*-test against the hypothetical value of 100 (* $p<0.05$, ** $p<0.01$, relative to control).

4.2. CB_2R activation triggers glucose uptake in the hippocampus

4.2.1. CB_2R activation increased glucose uptake in both wild-type and TgAPP mice

Basal glucose uptake in hippocampal slices of both WT mice and TgAPP mice was determined in a pairwise protocol similar to the above detailed. Both strains had similar values for basal glucose uptake (n=18, p>0.05).

In the WT and the TgApp mice, the selective CB_2R agonist JWH133 (1 µM), as well as the non-selective CB_1R/CB_2R agonist WIN (1 µM), significantly increased glucose uptake, when compared to vehicle (DMSO) (n=8, p<0.05) (Figure 9).

However, the blockade by DuP697 (500 nM) of COX-2, an enzyme responsible for anandamide degradation, augmented glucose uptake (n=6, p<0.05) only in the WT (n=6, p<0.05), but not in TgAPP mice (n=6, p<0.05) (Figure 9). The selective CB_2R antagonist AM630 (1 µM) had no effect per se. However, it fully prevented the effects of JWH133 and WIN in both strains and the effect of DuP697 in WT mice (Figure 9). Finally, LY2183240 (100 nM), a potent dual inhibitor of FAAH and FAAH-like anandamide transporter (FLAT), failed to change glucose uptake in the WT mice (Figure 9).

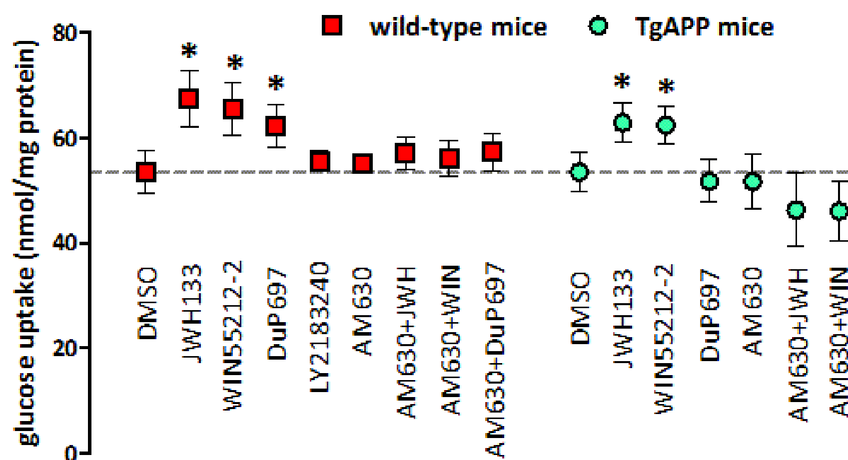

Figure 9. The CB_2R effect on glucose uptake in 300 µm-thick acute hippocampal slices of WT or TgAPP mice. CB_2R was activated by JWH133 (1 µM) or WIN55212-2 (1 µM) and inhibited by AM630 (1 µM); COX-2 was blocked by DuP697 (500 nM) and FAAH and FLAT were inhibited by LY2183240 (100 nM). Data represent the mean ± SEM of individual measurements (6≤n≤18) dashed line indicates the WT DMSO value (which was virtually identical to the TgAPP DMSO value). Statistically significant differences on glucose uptake were calculated by the one sample *t*-test against the DMSO controls (*p<0.05 vs. vehicle treated slices).

4.2.2. Glucose uptake in other rodent strains and its regulation by CB_2R

The basal rate of glucose uptake in hippocampal slices from young adult rats previously treated with Aβ (2 nmol, icv) did not change, when compared to control rats (18-day sham). In control young adult Wistar rats, JWH133 was effective only with the higher concentration (10 μM) (Figure 10). However, middle-aged C57Bl/6J mice showed increased glucose uptake in the presence of 1 μM JWH133 (n=10, p<0.05) (Figure 9). Similarly to the middle-aged C57Bl/6J mice, JWH133 significantly increased glucose uptake in hippocampal slices of young adult CD-1 mice (n=8, p<0.05) (Figure 10).

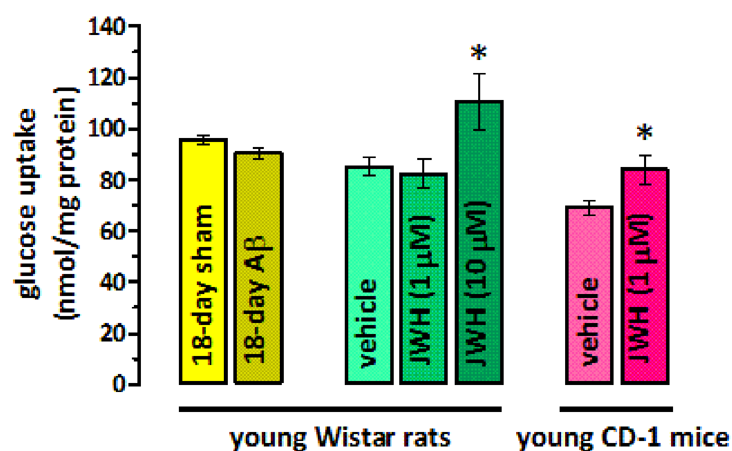

Figure 10. Basal and JWH133-stimulated glucose uptake values in acute hippocampal slices of young adult Wistar rats (young adult Wistar rats 18-days sham and 18-days after Aβ injection (yellow bars) and another set of young adult Wistar rats (green bars)) and of young adult CD-1 mice (purple bars). One-Way ANOVA with Bonferroni's post-hoc analysis failed to detect differences (p>0.05) in the rate of glucose uptake in control (DMSO-treated) slices throughout the following pairs of rodent groups: rat sham vs. control rat, control rat vs. control CD-1 mice control CD-1 mice vs. control C57Bl/6J. Data represent the mean ± SEM of individual measurements from 6-8 animals (*p<0.05).

4.2.3. CB_2R activation rapidly enhances fluorescent glucose uptake in hippocampal slices of young adult C57Bl/6 mice

Fluorescent glucose uptake assay allows better subregional and temporal resolution, i.e. the real-time observation of the accumulation of fluorescent deoxyglucose (2-NBDG) by fluorescent microscopy. In the superfused hippocampal slices of young adult C57Bl/6 mice, the accumulation of 2-NBDG signal show subregional differences. The greatest uptake was observed in the *stratum lacunosum*, followed by the *strata radiatum* and *moleculare*, and the smallest signal was found in the *strata pyramidale* and *granulare* (Figure 11). Thus, both CB_2R agonists JWH133 (1 μM) and GP1a (100 nM), increased 2-NBDG uptake, mainly concentrated in astrocyte-rich zones of the hippocampus, i.e. *strata lacunosum*, *radiatum*, and *moleculare* (Figure 11).

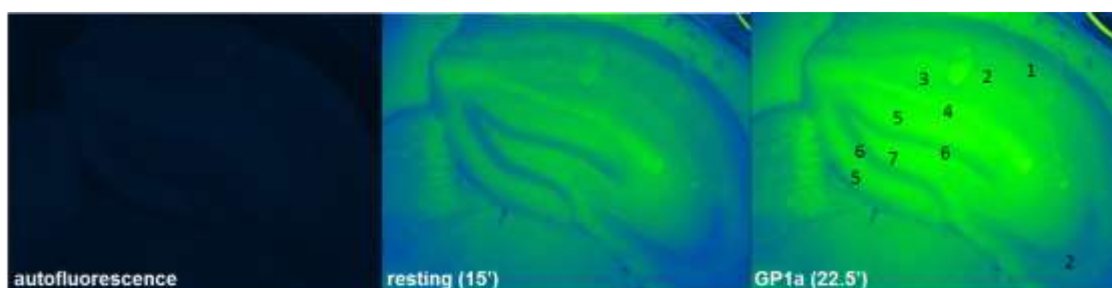

Figure 11. Time-course and subregional variation of the effect of CB_2R agonists on the uptake of the fluorescent glucose analogue 2-NBDG, in 300 μm-thick hippocampal slices of young C57Bl/6j male mice. The figure shows representative individual images, selected from an experiment, illustrating the slice autofluorescence and the distribution of fluorescent signal right before the treatment (minute 15 of baseline), and 7.5 min after adding GP1a (100 nM). The numbers mark the regions highlighted by their difference in 2-NBDG uptake: 1: stratum oriens 2: s. pyramidale (low intensity) 3: s. radiatum (high intensity), 4: s. lacunosum (greatest uptake activity), 5: s. moleculare (high intensity), 6: s. granulare (lowest signal), 7: hylus (high signal).

DMSO (0.1% v/v) tendentiously slowed the progressive increase of 2-NBDG signal, whereas the selective CB_2R agonists GP1a (100 nM) (Figures 11,12) and JWH133 (1 μM) rapidly increased the velocity of 2-NBDG accumulation in the total area of the slices (Figure 12). The figure 12B represents the significant increases in fluorescence intensity with GP1a (100 nM) ($p<0.01$ vs DMSO) or JWH133 (1 μM) ($p<0.05$ vs DMSO) in the post-treatment period.

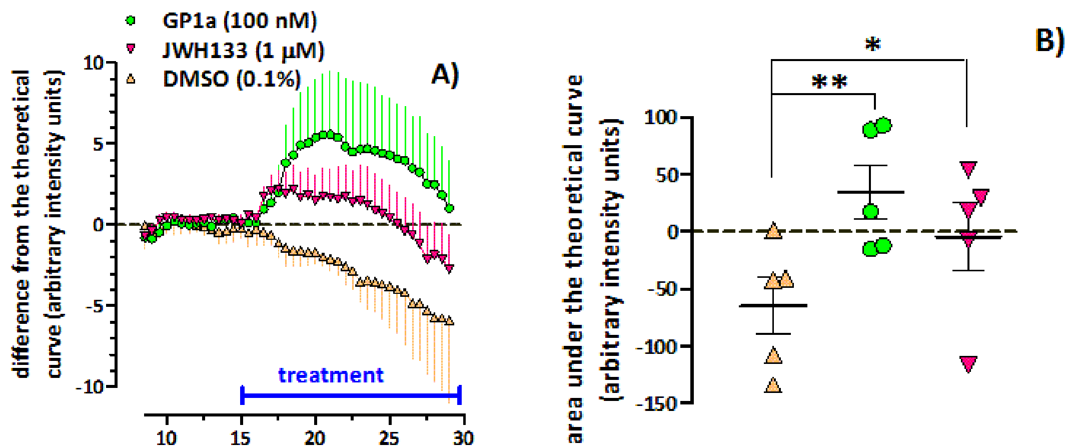

Figure 12. Time-course and fluorescence intensity variations of the effect of CB_2R agonists on the uptake of 2-NBDG, in 300 µm-thick hippocampal slices of young C57Bl/6j male mice. **A)** Changes in 2-NBDG uptake rate after the treatment with the vehicle DMSO (0.1%), GP1a (100 nM) or JWH133 (1 µM). Dashed line at zero represents the predicted velocity of 2-NBDG uptake if the slices were left untreated (theoretical curve). This indicates a tendency for DMSO to decrease the velocity of glucose uptake ($p>0.05$ with one-sample t-test). Curves represent the mean ± SEM of the averaged duplicate experiments from five mice. **B)** Scatter graph illustrates the individual amplitude variations in fluorescence intensity upon treatment with DMSO, JWH133 and GP1a, obtained in 5 animals in duplicate. The JWH133 and the GP1a groups were compared to the DMSO group with the help of Repeated Measures ANOVA followed by Dunnett's Multiple Comparison Test (*$p<0.05$, **$p<0.01$).

Chapter 5. Discussion

The ECS have been extensively investigated in the past two decades due to its involvement in human health and disease. The ECS as therapeutic target in metabolic and neurodegenerative disorders such as diabetes and AD (Matias et al. 2008; Lipina et al. 2012; Mulder et al. 2011) have been long recognized which led to the introduction of Acomplia (rimonabant, an antiobesity medicine) to the market in 2006 (Di Marzo & Matias 2005; Matias et al. 2008; Silvestri & Di Marzo 2013). My research has resulted in additional findings suggesting that the cerebral ECS may possess therapeutic potential to control both local cerebral and peripheral metabolic homeostasis.

It has been previously recognized that both peripheral and cerebral CB_1Rs are involved in systemic glucoregulation (Matias et al. 2008; Penner et al. 2013). Recently, we found that the density of hippocampal CB_1Rs is altered after STZ-injection in the rat (Duarte et al. 2007), and that CB_1R inhibit the TCA cycle in hippocampal neurons and astrocytes (Duarte et al. 2012), while the genetic ablation rather than the acute pharmacological blockade of CB_1Rs decreases the basal rate of hippocampal glucose uptake in mice (Lemos et al. 2012). These altogether indicate the intricate involvement of the endocannabinoid system in cerebral glucoregulation. Due to the therapeutic potential of these critical observations we decided to further investigate the roles of the ECS including those of the CB_1R and the CB_2R in cerebral glucoregulation.

GR activation causes accumbal insulin resistance via CB_1R activation

Brain glucose uptake is not dependent on insulin. However, in certain brain areas, insulin may mediate glucose uptake by an indirect mechanism as a result of the neuronal activity modulation (Vogt & Brüning 2013). In this manner, insulin increases GABAergic neurotransmission (Wan et al. 1997; Jin et al. 2011), thereby decreasing cortico-hippocampal activity in humans (Mielke & Wang 2005). In fact, this suggests that insulin may regulate reward-related feeding behavior through a postprandial feedback mechanism, on the mesolimbic circuitry (Speed et al. 2011; Vogt & Brüning 2013).

In my thesis work, we regard accumbal glucose uptake as a measure of accumbal activity. We used a relatively high concentration of insulin (300 nM) to guarantee that

even if the glassware and plastic tubes absorb insulin from the medium, enough peptide remains in solution to rapidly saturate the slices (Goebel-Stengel et al. 2011). However, most *in vitro* studies in brain preparations use hundreds of nanomolars of insulin (Labouèbe et al. 2013) and insulin levels are 10-100-times higher in the brain than in the plasma (Ghasemi et al. 2013). Insulin (300 nM) acutely increases glucose uptake in rat accumbal slices, which was also demonstrated in the NAc of humans *in vivo* (Anthony et al. 2006). This is in concert with that the ECS is involved in the control of food intake, stimulating the preference for sweet and tasty food, through the modulation of the activity of hypothalamus, brainstem and mesolimbic system, including the VTA and the NAc (Soria-Gómez et al. 2007; Matias et al. 2008; Silvestri & Di Marzo 2013).

CB_1R activation by WIN55212 per se had no effect on the basal glucose uptake but it prevented the action of insulin when combined. In turn, when we blocked the CB_1R by O-2050, glucose uptake also increased indicating the presence of some tonic inhibitory endocannabinoid action. O-2050 and insulin failed to produce additive effect indicating a mutual occlusion between the two actions, suggesting convergent mechanisms of action. Moreover, it was found recently that CB_1R form a heteromeric complex with IRs in pancreatic β-cells (Kim et al. 2012) and also with RTKs in neuronal cells (Dalton & Howlett 2012). Thus, our results suggest that there is a possible interaction of these receptors in the brain.

IGF1R can regulate many insulin actions in the brain and also may be more important than insulin in the regulation of cerebral glucose uptake, at least during development (Russo et al. 2005). Our results indicated that IGF1R blockade by I-OMe-Tyr had no effect on insulin-induced glucose uptake in NAc slices, supporting the idea that IR, and not IGF1R, is responsible for insulin-stimulated glucose uptake.

The glucocorticoid DEX impaired the effect of insulin in the accumbal slices. This result is in agreement with previous studies, which indicate that glucocorticoid excess leads to insulin resistance through the inhibition of insulin signaling (Lansang & Hustak 2011; Di Dalmazi et al. 2012). Moreover, it is known that a sub-acute and chronic administration of DEX in healthy individuals leads to insulin resistance, hyperinsulinemia, and impaired glucose tolerance (Nicod et al. 2003). Previous reports suggested an involvement of a hypersensitivity to glucocorticoids, or an increase of

their circulating levels, with the induction of food intake and the development and maintenance of obesity (Tataranni et al. 1996; Zakrzewska et al. 1999). Additionally, this is associated with a permanent alteration in the activity and metabolism of dopaminergic areas, including the NAc, which may have a role in stress-induced impairment in the reward area (Bock et al. 2012; Shpilberg et al. 2012; Barik et al. 2013).

However, the pathomechanism whereby glucocorticoids impair insulin signaling is still unknown. O-2050 in the presence of DEX was still capable of increasing glucose uptake, and CB_1R blockade also rescued insulin's action. Thus, this suggested that CB_1R blockade prevented the effect of DEX in the impairment of insulin's action in the NAc. Accordingly, previous findings suggested that the ECS is responsive to modulation by both stress and glucocorticoids, within the hypothalamus and limbic structures. Moreover, CB_1R signaling is involved with the rapid effects of glucocorticoids (Hill & McEwen 2010; Ko et al. 2012; Atsak et al. 2012). The modulation of ECS may occur through the binding of these hormones to a G protein-coupled receptor, activating an intracellular signaling pathway, which induces the synthesis of eCBs in the brain (Di et al. 2003). Besides, there are several pieces of evidence of a decrease of eCB levels and CB_1R expression, under a prolonged exposure to stress and/or glucocorticoids (Marco et al. 2011), suggesting a biphasic relationship between the ECS and stress (Wang et al. 2012).

The endocannabinoid 2-AG is synthesized on demand by DAGLα in the nervous system (Alger & Kim 2011; Castillo et al. 2012). Hence, it is expected that 2-AG signaling is associated with greater energy expenditure, as well as, the increase of glucose consumption under brain activity (Pellerin & Magistretti 2012). Moreover, previous findings demonstrated in rat models, that stress appears to mobilize 2-AG signaling in a variety of limbic structures (Hill & McEwen 2010). In this manner, we investigated how alterations in 2-AG levels could affect NAc glucose uptake.

Although CB_1R blockade by O-2050 produced facilitator effect on glucose uptake, this was not seen by the removal of the endogenous CB_1R agonist 2-AG beyond tendency, indicating that another endocannabinoid, probably anandamide also tonically activated the CB_1R. However, in the presence THL, DEX failed to impede insulin to increase glucose uptake. Hence, dexamethasone-induced insulin resistance is

prevented by the blockade of either the CB_1R or of 2-AG synthesis. As expected, an up-regulation of 2-AG levels through the inhibition of 2-AG metabolism prevented insulin from stimulating glucose uptake. These results support the idea that dexamethasone produces the release of the endocannabinoid, 2-AG, and in turn, 2-AG prevents the action of insulin. These are in concert with previous findings of DAG-induced insulin resistance (Amati 2012). This may suggest that the glucocorticoid-induced DAGL activation and the consequent 2-AG release activate the CB_1R which is an inhibitory co-partner of the IR. The scheme below represents the proposed mechanism for the DEX-induced insulin action impairment through the ECS (Figure 13).

Figure 13. Schematic diagram of the proposed model for the DEX-induced insulin-resistance. The activation of the glucocorticoid receptor (GR) by glucocorticoids (stress and hypercortisolemia) stimulates 2-AG synthesis through DAGLα activity. 2-AG activates CB_1R which inhibits insulin's action.

Thus, mechanisms that lead to increased endocannabinoid signaling including chronic stress and Cushing-syndrome will impair insulin actions, may be involved in the onset of neurological disorders such as AD, obesity and type 2 diabetes (Sonino et al. 1998; Lansang & Hustak 2011; Solas et al. 2013). In this manner, blockers of CB_1R would be beneficial to combat over feeding behavior, obesity and diabetes. For this

purpose, the CB_1R antagonist rimonabant (Acomplia) was marketed by Sanofi in 2006 (Matias et al. 2008).

Rimonabant is an example of an antagonist/inverse agonist of CB_1R, which proved to be helpful in the reduction of food intake leading to reduced body weight and also able to ameliorate obesity-associated metabolic syndrome (Jbilo et al. 2005; Thornton-Jones et al. 2006; Després 2007). Unfortunately, rimonabant was banned from the market in 2008 due to exerted unacceptable side effects, causing anxiety, depression and even instigate suicide in patients (Kang & Park 2012; Kirilly et al. 2012).

Recently it was shown that rimonabant's side effects are attributed to the inverse agonism at CB_1R. In contrast, silent CB_1R antagonists such as O-2050 and NESS0327 did not cause anxiety or anhedonia in the first in vivo experiments and yet, efficiently reduced food intake and weight gain (Meye et al. 2012), envisaging a new class of antiobesity medicines.

It is known that the nutritional state (e.g. fasted versus fed) and different food stimuli can alter the activity of brain reward systems demonstrating a interaction between homeostatic and hedonic features of feeding behavior with biasing fasting reward systems towards high-calorie foods (Goldstone et al. 2009). In our results, insulin (300 nM) significantly reduced accumbal glucose uptake in fasted rats. Although this experiment seems paradoxical for the first sight, in fact, after severe fasting, more food intake is necessary than during a normal meal. Hence, the first rise of plasma insulin levels should not trigger the same satiety response as normally to prevent the termination of the food intake. Hence, our data supports the notion that insulin may have a complex role as modulator of the satiety response.

Indeed, dopamine, insulin and the PKB/Akt signaling are intricately intertwined in the mesolimbic area (Garcia et al. 2005; Williams et al. 2007; Speed et al. 2011). As already mentioned, PKB/Akt is a key element in the insulin and growth factors signaling pathways and this kinase is also involved in feeding behavior, regulating DA signaling and homeostasis (Speed et al. 2011). Thus, it is expected that the ability of reward circuits to respond to insulin can be impaired in insulin resistant states, such as in obesity and prediabetes (Figlewicz et al. 2008; Egecioglu et al. 2011).

Insulin availability in the brain depends on its ability to cross the BBB from the peripheral circulation, although the controversy about a small insulin production in

CNS still exists (Santos et al. 1999; Banks 2004). Nevertheless, insulin effects in CNS depend mostly from circulating insulin. In STZ-induced diabetic rats, the accumbal effect of insulin is impaired indicating a maladaptive pathological alteration which requires further investigation.

Cerebral role of CB_2R in the hippocampus: stimulation of glucose uptake

Since it is known that diabetes and AD have much in common including the impairment in insulin signaling and glucose metabolism (Correia et al. 2012; Jolivalt et al. 2012), we aimed at mapping how ECS can influence glucose or insulin-mediated glucose uptake.

Although it was suggested that the cerebral glucose metabolism could be altered in this disease, the lower ^{18}FDG signal detected in AD patients only measures the rate of the uptake instead of the metabolism of the metabolically resistant glucose analogue ^{18}FDG (Martín-Moreno et al. 2012). Since acute CB_1R activation affects only glucose metabolism (Duarte et al. 2012) rather than ^{3}H-deoxyglucose (^{3}HDG) uptake in the rodent hippocampus (Lemos et al. 2012) we turned now to the CB_2R to map if it is involved in the control of glucose uptake. Moreover, CB_2R density is positively correlated with the severity of Aβ pathology rather than CB_1R (Esposito et al. 2008; Solas et al. 2013) implicating that CB_2Rs may serve as a better therapeutic target than CB_1Rs to control the pathology of AD.

Our results indicate that the activation of CB_2R by a selective (JWH133) and a non-selective (WIN) CB_2R agonists stimulated glucose uptake in acute hippocampal slices of both middle-aged WT and TgAPP mice. The concentration of agonists (1 µM) was already maximal to activate the receptors but still, substantially smaller responses were detected to both JWH133 and WIN in TgAPP mice, indicating a tendency for decreased CB_2R sensitivity in this model. The effect of the agonists was prevented by the selective CB_2R antagonist, AM630, which alone had no effect. Notably, CB_2R activation increased glucose uptake by 1/3-1/5 over the basal rate, which is a considerable increase because basal activity represents ~90% of brain metabolism (Magistretti 2006).

The eCB anandamide has an antagonistic relationship with Aβ which appears to be bi-directional: *in vitro* Aβ-toxicity is prevented by anandamide in human cell lines (Milton, 2002), and vice versa, Aβ-treatment decreases anandamide levels in C6 rat astroglioma cells (Esposito et al. 2007). Accordingly, anandamide levels decrease in the AD brain and inversely correlate with Aβ levels (Jung et al. 2012). COX-2 is a major metabolizing enzyme for anandamide in the mouse brain (Glaser & Kaczocha 2010) and β-amyloidosis induces COX-2 expression in astrocytes (Giovannini et al. 2002) and in AD-affected neurons; this renders COX-2 inhibition helpful to decrease the rate of incidence or to slow the progress of AD (Berk et al. 2013).

Our results suggested that COX-2 inhibition by its selective antagonist, DuP697 (Gierse et al. 1995), facilitated glucose uptake via CB_2R activation which was observed in the WT mice but not in the TgAPP mice. This pharmacologic intervention was also sensitive to the selective CB_2R antagonist AM630. This suggests that anandamide levels were (physiologically) suppressed by COX-2 only in the WT mice according to previous reports (Glaser & Kaczocha 2010; Pamplona et al. 2010; Straiker et al. 2011). It is hence more likely that the synthesis rather than the metabolism of anandamide is altered in TgAPP mice. In anterior studies, where TgAPP 2576 mice were orally treated for four months with the CB_2R-selective agonist JWH133, AD phenotype was attenuated, including recovery from memory impairment and tissue pathology and the normalization of pathologically increased COX-2 levels (Martín-Moreno et al. 2012).

Furthermore, the dual FAAH and FLAT inhibitor, LY2183240 lacked effect on glucose uptake, indicating that either the uptake of anandamide or its metabolism by FAAH do not play a significant role in cerebral glucoregulation in the WT mice. Indeed, anandamide production in cortical structures requires the concurrent stimulation of both NMDA and acetylcholine receptors (Stella & Piomelli 2001), both of which become hypofunctional with β-amyloidosis (e.g. AD) (Pavía et al. 1998; Giovannini et al. 2002), thus prompting an impaired anandamide synthesis. This may explain why COX-2 blockade is not capable to trigger glucose uptake in TgAPP mice (Figure 14).

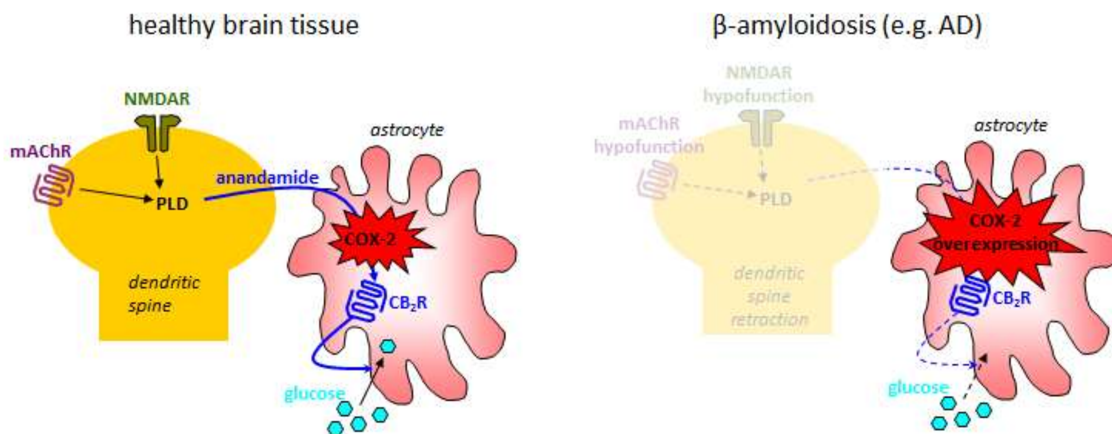

Figure 14. Schematic diagram of the hypothetic glucoregulator control of anandamide in healthy rodents and under β-amyloidosis. Anandamide is produced in neurons upon muscarinic and NMDAergic stimuli, but is also metabolized rapidly by astrocytic COX-2. Thus, COX-2 blockade can trigger CB_2R activation and glucose uptake in the healthy brain tissue.

Since there was a lack of effect by the CB_2R antagonist, AM630 in WT mice, this suggests a lack of tonic stimulation of glucose uptake by these receptors. Together with our others results, it is suggested that the tonic stimulation of glucose uptake is due to the basal metabolism of anandamide by COX-2. Hence, if Aβ accumulation impairs anandamide synthesis it will have no direct consequence on basal glucose uptake rates - and this is what we observed in both the chronic transgenic mouse and the Aβ-injected rat model. There was no difference in glucose uptake between control and young adult rats previously treated with Aβ, indicating that the glucoregulator role of CB_2Rs was age or strain/species-dependent. The CB_2R activation by JWH133 (1 µM) increased glucose uptake in young adult CD-1 mice, similar to the middle-aged C57Bl/6J mice. In control young Wistar rat, it was observed that a concentration of 1 µM of JWH133 had no effect and only a higher concentration (10 µM) stimulated glucose uptake, indicating some species' differences.

CB_2Rs are present in most brain cell types, including activated microglia and astrocytes controlling neuroinflammation (Halleskog et al. 2011; Ashton & Glass 2007), and astrocytoma, inhibiting its growth (Sánchez et al. 2001; Cudaback et al. 2010). These receptors are also expressed in neurons (Atwood & Mackie 2010) and they are present in cortical, hippocampal, brain stem, striatal and other neurons, either in the soma or in the nerve terminals, modulating neuronal communication (Van Sickle et al. 2005; Lanciego et al. 2011; Andó et al. 2012; Callén et al. 2012; den Boon et al. 2012).

However, the cellular site where CB_2R exert their glucoregulatory actions remains to be defined. Our results indicated that CB_2R activation by both agonists JWH133 and GP1a, increased 2-NBDG uptake in hippocampal slices of young adult C57Bl/6 mice.

This 2-NBDG uptake increase was mainly concentrated in astrocyte-rich zones of the hippocampus, i.e. *strata lacunosum*, *radiatum*, and *moleculare* than in neuronal cell body-rich zones such as the *strata pyramidale* and *granulare*. This supports previous studies reporting that 2-NBDG is preferentially taken up by astrocyte-rich areas than neuronal areas (Jakoby et al. 2013), although it can be taken up by both neurons and astrocytes in culture (Abeti et al. 2011). This result does not exclude a glucoregulating role for neuronal CB_2R. Hence, CB_2R may either directly stimulate glucose uptake by changing intracellular calcium waves and the activity of the circuitry (den Boon et al. 2012) or CB_2R may rescue CB_1R-mediated suppression of glucose uptake since CB_1R activation attenuates glucose metabolism in both astrocytes and neurons (Duarte et al. 2012) and CB_1R and CB_2R form heterodimers in the brain, exerting negative cross-talk on their common signaling pathway (Callén et al. 2012). Further studies should also detail the mechanisms underlying this CB_2R glucoregulation in brain.

Our results further support the idea of CB_2R agonists as a novel class of nootropics since glucose facilitates cognition and memory in human (Messier 2004), and metabolic boosting alleviates the cognitive symptoms of dementias (Branconnier 1983). These receptors have also gained attention as attractive therapeutic targets for the regulation of food intake, eating disorders, pain management and immune system modulation (Ishiguro et al. 2010; Atwood & Mackie 2010).

The advantages of therapeutic targeting CB_2Rs over the manipulation of the most abundant CB_1Rs are the lack of psychoactivity of CB_2Rs. A long-term exposure to CB_1R agonists triggers different adverse psychoactive effects in different brain cells, whereas CB_2Rs activation appears to affect specific targets and processes (Ashton & Glass 2007; Atwood & Mackie 2010; Pertwee 2012). Another advantage is due to the expression changes in disease models: cortical CB_1R density does not increase or even decreases in AD (Ramírez et al. 2005; Mulder et al. 2011; Solas et al. 2013) whereas CB_2Rs density increases in AD and Down-syndrome, as well as in vitro β-amyloidosis (Esposito et al. 2007; Ruiz-Valdepeñas et al. 2010; Halleskog et al. 2011; Solas et al. 2013). This likely

represents a self-defense process as CB_2Rs activation confers neuroprotection in several experimental models (Ramírez et al. 2005; Esposito et al. 2007; Ashton & Glass 2007; Ruiz-Valdepeñas et al. 2010; Martín-Moreno et al. 2012).

Chapter 6. Conclusions

In these two lines of investigations we addressed the multifaceted role of the endocannabinoid system in the regulation of cerebral glucose uptake. The ECS is deeply involved in the control of food intake and food-induced satiety, e.g. through the modulation of NAc activity. Moreover, insulin is now being suspected to mediate feeding-related reward, and thus, may create a postpandrial feedback mechanism. Hence, we wanted to investigate whether insulin affects (increases) accumbal glucose uptake and if this purported action of insulin was dependent on local CB_1R signaling. Our results indicate that these were indeed the case. Interestingly, CB_1R activation counteracted the stimulation of glucose uptake, i.e. it produced a negative effect, and the endocannabinoid likely to be involved in CB_1R activation appeared to be 2-AG.

Interestingly, these findings were fully complementary in the hippocampus, where the other cannabinoid receptor, the CB_2R and the another major endocannabinoid, anandamide were found to be involved in the control of glucose uptake. Their effect was positive, corresponding to the stimulation of glucose uptake.

Altogether, these indicate that both (in)direct CB_1R blockade and (in)direct CB_2R activation may prove to be beneficial to combat impaired brain functions involving - in part - insulin resistance, such as type-2 diabetes and AD. To explore these possibilities, additional studies are necessary.

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