



NEUROINFLAMMATION AND INSULIN
RESISTANCE UNDERLY COGNITIVE IMPAIRMENT
SEX DIFFERENCES AND POTENTIAL
TREATMENTS

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SUMMARY

Neuroinflammation and type 2 diabetes mellitus (T2DM) are two correlated processes. T2DM patients display insulin resistance: there are normal levels of insulin but the response to it is reduced. Brain insulin resistance and neuroinflammation are important factors contributing to the onset of neurodegenerative diseases, such as Alzheimer's disease (AD). Therefore, molecules that are able to inhibit neuroinflammation could be effective for the prevention and/or treatment of neurological diseases of inflammatory aetiology. Abscisic acid (ABA) is a phytohormone involved in the plant response to hydric stress situations. This compound has also been found in mammals. ABA had been demonstrated to have peripheral anti-inflammatory effect and insulin sensitizing properties. Thus, we hypothesised that ABA chronic administration could have protective effects in a model of neuroinflammation induced by high fat diet (HFD). We confirmed in our model that ABA counteracts peripheral insulin resistance, improving glucose tolerance in HFD-fed animals. Regarding cognitive function, ABA administration rescued the memory alterations observed in HFD-fed rats. Moreover, we observed that ABA effectively prevented the HFD-induced increase in pro-inflammatory markers (TNF- α , APP -Amyloid Precursor Protein- expression and reactive microglia). Furthermore, treatment with ABA counteracted the HFD-induced reduction in BDNF (brain-derived neurotrophic factor) mRNA expression and hippocampal neurogenesis.

Alterations of the insulin receptor (IR) and the insulin substrates (IRS) function is proposed to underlie insulin resistance. In this thesis, we analyzed the effect of HFD and ABA on the expression of substrates 1 and 2 (IRS1, IRS2) in hippocampus. The model of neuroinflammation induced by HFD did not affect the IR, reduced IRS2 and increased IRS1 mRNA levels. This result suggests that IRS1 and IRS2 can be differentially affected by neuroinflammatory processes. Furthermore, ABA restored the HFD-induced changes in IRS1 and IRS2 levels.

In summary, data given in this thesis demonstrate the therapeutic effects of ABA, postulating this phytohormone as a nutraceutical alternative to current anti-inflammatory drugs with adverse effects.

The attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that affects children although the affection can last through adulthood. Inflammation has been proposed a contributing factor in this disorder, since ADHD patients display high levels of pro-inflammatory cytokines in blood. In an internship at the University of Bordeaux, I studied the anti-inflammatory effects of ABA in an ADHD mouse model produced by 6-OHDA neonatal lesion. In this pilot study, female and male mice seem to display differences in ADHD symptomatology. Furthermore, ABA administration appeared to also affect differentially males and females, almost opposite in certain parameters like pain sensitivity. Although these preliminary data need to be confirmed (by increasing the subjects) this pilot study points to a revision of disease preclinical models and pharmacological treatments in comparative females and males studies.

The specific and distinct role of IRS1 and IRS2 in distinct brain networks, isolated from other comorbidities such as chronic inflammation or AD linked genetic mutations, has not been fully established. To that end, we have used of adeno-associated virus (AAV) expressing short hairpin ribonucleic acid (shRNA) targeting IRS1 (shIRS1) into the dorsal HPC of female and male rats. We found that female rats inoculated with shIRS1 showed spatial and recognition memory deficits but males only showed deficits in the spatial memory task. This result suggests that the female brain may display a higher susceptibility to insulin resistance caused by IRS1 deficits. In post-mortem analysis, we observed that the viral particle had mainly infected somatostatin (SOM)-positive and parvalbumin (PV)-positive neurons in the hippocampal dentate gyrus. Accordingly, we found AAV fibres in the medial septum (MS) from the long projecting SOM hilar neurons. In the MS, we observed that synaptophysin labelled puncta on shIRS1 AAV fibres were reduced compared to control, thus suggesting an impaired synaptic plasticity due to IRS1 reduction. Hippocampal SOM regulation of septal PV neurons is crucial in the regulation of septo-hippocampal circuitries in memory formation. Our data shows that IRS1 is required for proper neuronal activity, since in the absence of neuroinflammation IRS1 knockdown can cause cognitive deficits.

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ABBREVIATIONS LIST

5-HT 5-hydroxytryptamine (serotonin)

6-OHDA 6-hydroxydopamine

A

A β Amyloid-beta

AAV Adeno-associated virus

ABA Abscisic acid

Acb Nucleus accumbens

AD Alzheimer's disease

ADHD Attention deficit hyperactivity disorder

AgRP Agouti-related peptide

AMPA α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPK Adenosine monophosphate-activated protein kinase

ANOVA Analysis of variance

APOE Apolipoprotein E

APP Amyloid-beta precursor protein

B

BAX Bcl-2-associated X

BBB Blood-brain barrier

BDNF Brain-derived neurotrophic factor

C

CA Cornu Ammonis

CA1 Cornu Ammonis 1

CA3 Cornu Ammonis 3

cADPR Cyclic adenosine difosfato-ribose

cAMP Cyclic adenosine monophosphate

CAR Cliff avoidance reaction

CaRT Cocaine- and amphetamine-regulated transcript

Cb Cerebellum

CB Calbindin

ChAT Choline acetyltransferase (Cholinergic)

CNS Central nervous system

CPu Caudate putamen

CR Calretinin

CREB cAMP response element-binding

CRH Corticotrophin-releasing hormone

CSF Cerebrospinal fluid

D

DA Dopamine

DAB 3,3'-diaminobenzidine

DAMPs Damage-associated molecular patterns

DAT Dopamine transporter

DCX Doublecortin

DG Dentate gyrus

DI Discrimination index

DPX Dibutylphthalate polystyrene xylene

DS Dorsal subiculum

E

EC Entorhinal cortex

EGFP Enhanced green fluorescent protein

ER Endoplasmic reticulum

ERK Extracellular signal-regulated kinase

F

f Fornix

FoxO Forkhead box O

FST Forced swim test

G

GABA Type A γ -aminobutyric acid

GFAP Glial fibrillary acidic protein

GL Granular layer

GLP1 Glucagon-like peptide 1

GLUT4 Glucose transporter 4

GLUT8 Glucose transporters 8

GLUTs Glucose transporters

GPCR G-protein coupled receptor

Grb Growth factor receptor-binding protein

GSK3 β Glycogen synthase 3-beta

H

HDB Horizontal limb diagonal band

HEK Human embryonic kidney cells

HFD High fat diet

HIL Hilar somatostatin interneurons with long axons

HIPP Hilar somatostatin interneurons

HPC Hippocampus

HPLC High-performance liquid chromatography

Hyp Hypothalamus

I

Iba1 Ionized calcium-binding adaptor molecule 1

icv Intracerebroventricular

IDE Insulin-degrading enzyme

IFN- γ Interferon gamma

IGF-1 Insulin-like growth factor-1

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

IKK β Inhibitor of nuclear factor kappa-B kinase subunit beta

IL Interleukin

IN Intranasal

IP Intraperitoneal

IR Insulin receptor

IRed Infrared

IRS Insulin receptor substrates

J

JNK c-Jun N-terminal kinase

K

KRLB Kinase regulatory-loop binding domain

L

LANC Lanthionine synthetase component C

LANCL2 Lanthionine synthetase component C-like protein 2

LEC Lateral entorhinal cortex

LPS Lipopolysaccharides

LTD Long-term depression

LTP Long-term potentiation

M

M1	Pro-inflammatory macrophage phenotype
M2	Anti-inflammatory macrophage phenotype
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MDD	Major depressive disorder
MEC	Medial entorhinal cortex
MEK	Mitogen-activated protein kinase
MM	Mammillary bodies
mL	Mililiter
ML	Molecular layer
MS	Medial septum
mTOR	Mammalian target of rapamycin
mTOR-S6K	Mammalian target of rapamycin-ribosomal protein S6 kinase
mTORC1	Mammalian target of rapamycin complex 1

N

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRP3	Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide

NOD Nucleotide-binding oligomerization domain-like receptor

NOR Novel object recognition

NPY Neuropeptide Y

NSAIDs Non-steroidal anti-inflammatory drugs

P

P85 Regulatory subunit of phosphoinositide 3-kinase

pAKT Phosphorylated AKT

PAMPs Pathogen-associated molecular patterns

PBS Phosphate buffered saline

PC Perirhinal cortex

PD Parkinson's disease

PDK1 3-phosphoinositide-dependent protein kinase 1

pERK Phosphorylated extracellular signal-regulated kinase

PFA Paraformaldehyde

PFC Prefrontal cortex

pfp Perforant pathway

PH Pleckstrin homology

PI3K Phosphatidylinositol 3-kinase

pIKK Phosphorylated inhibitor of nuclear factor kappa-B kinase subunit

PIP2 Phosphatidylinositol 4,5-bisphosphate

PIP3 Phosphatidylinositol (3,4,5)-trisphosphate

PKA Protein kinase A

PKC Protein kinase C

PoDG Polymorphic area of the dentate gyrus

POMC Proopiomelanocortin
PPAR γ Peroxisome proliferator-activated receptor gamma
PRRs Pattern recognition receptors
PS1 Presenilin 1
PSD-95 Postsynaptic density protein 95
pTAU Phosphorylated Tau
PTB Phosphotyrosine binding
PV Parvalbumin

R

RA Retinoic acid
RAR α Retinoic acid receptor alpha
RN Raphe nucleus
RT Room temperature
RXRs Retinoid X receptors

S

sc Subcutaneous
SD Standard diet
SEM Standard error of mean
Ser Serine
SGZ Subgranular zone
SH Src homology domain
SH2 Src homology 2 domain
Shc Src homology and collagen
shIRS1 Short hairpin ribonucleic acid targeting insulin receptor substrate 1
shRNA Short hairpin ribonucleic acid
SHP-2 Src homology domain-containing phosphatase-2

SHPTP-2 Src homology 2 domain protein tyrosine phosphatase
Sol Solitary tract nucleus
SOM Somatostatin
SOS Son-of sevenless
SuM Supramammillary nucleus
Synap Synaptophysin

T

T1DM Type 1 diabetes mellitus
T2DM Type 2 diabetes mellitus
TB Tris buffer
TBS Tris-buffered saline
Thr Threonine
TLRs Toll-like receptors
TNF α Tumor necrosis factor alpha
TNFR Tumor necrosis factor receptor
TrkB Tropomyosin receptor kinase B
Tyr Tyrosine
TZDs Thiazolidinediones

V

VDB Vertical limb diagonal band
VEH Vehicle
VTA Ventral tegmental area

1

GENERAL INTRODUCTION

1.1. Insulin signalling in brain: receptor and substrates

1.1.1. Insulin transport and receptor functions

Since brain can use glucose independently of insulin, brain was considered for a long time an insulin insensitive tissue. However, the discovery of the insulin receptor (IR) in the 70's^[1] changed this view, and nowadays, insulin signalling important role in brain function is widely recognized. Insulin is an endocrine hormone mainly synthesized and secreted by the pancreatic β -cells^[2]. The source of brain insulin was debated for a long time, but now accumulated evidence shows that, although pancreatic insulin crosses the blood-brain barrier (BBB) by means of a saturable transporter there is also local insulin synthesis^[3]. The insulin transporter is localized in the brain capillary endothelial cells, in this way insulin crosses from blood plasma to brain interstitial fluid and then to cerebrospinal fluid (CSF)^[4]. Insulin transport through the BBB can be affected by different conditions, as has been reported in rodent models of diabetes^[5] and aging^[6]. Insulin transport rate may also depend on the brain area. Thus, early studies suggested that the olfactory bulb has very high insulin transport and insulin degradation rate^[7], also the hypothalamus (Hyp) and cortex^[8], whereas no detectable transport reported in thalamus, midbrain or occipital cortex^[8].

Peripherally, the principal role of insulin is to induce blood glucose uptake into muscle adipose tissue and liver, after food intake when there is a glucose increase in blood^[10]. Glucose uptake is mediated by the transmembrane glucose transporters (GLUTs). The main insulin-sensitive glucose transporters are GLUT4 and the more recently discovered member, GLUT8. Once insulin stimulates its receptor, insulin-dependent glucose transporters are translocated to the plasma membrane by an Akt2-mediated signalling. The expression of GLUT4 is reduced in the adipose tissue of type 2 diabetes mellitus (T2DM) patients, contributing to reduced glucose uptake by these cells^[11].

Centrally, GLUT4 co-expresses with IR in Hyp, cerebellum^[12], and hippocampus (HPC), where it has been demonstrated that the mechanism of translocation is similar to peripheral, through Akt cascade^[13]. GLUT4 seems to be specifically expressed in neuronal soma and dendrites. At least in rat HPC, GLUT4 labelling has only been found in neuronal but not in glial cells^[14]. Moreover, GLUT4 expression seems specific of some types of neurons. For example, in rat basal forebrain, cholinergic (ChAT) and parvalbumin (PV), but no calbindin (CB) and calretinin (CR) neurons, express GLUT4^[15]. On the other hand, GLUT8 is distributed widespread in the rat brain^[16]. Similarly to GLUT4, GLUT8 was found specifically in the cell body and proximal apical dendrites of hippocampal rat neurons but not in astroglia or microglial cells^[16]. Unlike GLUT4, GLUT8 is not expressed in the plasma membrane but in endoplasmic reticulum (ER), endosomes and lysosomes membranes. Interestingly, elevated plasma glucose levels increases GLUT8 association with ER, providing glucose availability for protein glycosylation in the ER lumen^[17,18].

One of the best well-known insulin effects in brain is the regulation of food intake and energy balance. In the hypothalamus, insulin downregulates orexigenic peptides: neuropeptide Y (NPY) and Agouti-related peptide (AgRP). Simultaneously, insulin upregulates the anorexigenic peptides: proopiomelanocortin (POMC), and the cocaine-amphetamine-regulated transcript (CaRT). Thus, the disruption of IR signalling results in increased appetite and food intake, leading to obesity^[19,20].

Most relevant to this thesis, brain insulin resistance and metabolic syndrome leads to accelerated cognitive decline and increases the risk of developing Alzheimer's disease (AD)^[21]. Neuroimaging studies in T2DM patients displayed hippocampal and amygdala atrophy together with altered functional connectivity with other brain regions^[22-24].

Preclinical studies with diabetic rats show long-term potentiation (LTP) (see note¹) alterations due to diminished expression of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors at the postsynaptic density [25]. Conversely, insulin has been shown to induce LTP in mouse hippocampal slices [26]. The upregulation of LTP can be due to insulin action on postsynaptic proteins, thus, insulin administration can increase NMDA receptor trafficking to postsynaptic membrane [27], and stimulate tyrosine (Tyr) phosphorylation in NR2A and NR2B subunits [28]. Furthermore, in rat hippocampal slices, insulin stimulates the postsynaptic density protein 95 (PSD-95) translation to membrane, through the phosphatidylinositol 3-kinase PI3K-Akt-mammalian target of rapamycin (mTOR) signalling pathway [29]. Furthermore, insulin can also affect long-term depression (LTD) (see note²), via promoting the AMPA receptor endocytosis [30]. In addition to glutamatergic transmission, insulin can also modulate inhibitory synapses through type A γ -aminobutyric acid (GABA) receptor activity [31].

Not surprisingly, reducing hippocampal IR expression disrupts the neuroplasticity leading to spatial memory deficits [32]. Furthermore, insulin inoculation in brain can reverse the alterations in spatial memory caused by inflammation, in lipopolysaccharides (LPS)-inoculated rats [33] and in a chronic stress model [34]. Also in clinical studies, intranasal (IN) insulin administration for 4 months was shown to improved delayed memory and preserved general cognitive function in AD patients [35].

IR activity can also affect psychiatric symptoms (i.e. mood disorders) by the modulation of dopamine (DA) [36] and serotonin (5-HT) signalling [37], either via upregulation of the monoamine oxidase activity (enzyme that degrade monoamines) [38] or by increasing dopamine transporter (DAT) mRNA expression [39]. Furthermore, animals suffering type 1 diabetes mellitus (T1DM) or T2DM showed depressive-like behaviour and insulin sensitizers administration can counteract these symptoms [40].

Moreover, there is also insulin signalling in glial cells. Insulin stimulation in astrocytes promotes proliferation [41] and glutamate receptor expression [42], indicating that these are insulin-sensitive cells. In rat HPC, intracerebroventricular (icv) insulin infusion induce an increase in Akt Ser473 phosphorylation in microglia cells [43].

However, despite the role of insulin and the deleterious effects of brain insulin resistance, the excess of insulin, hyperinsulinemia is also toxic to neurons and glia cells. For that reason, it is crucial to maintain optimal insulin sensitivity, in other to prevent hyperinsulinemia.

The IR is a transmembrane Tyr kinase receptor which comprises two α and two β subunits. The α subunit contains the extracellular hormone-binding site and the β subunit has a transmembrane domain and the intracellular Tyr kinase domain. Each IR can bind up to two or three insulin molecules, the first one with higher affinity than the rest. Insulin has a negative feedback effect being its own signalling regulator. Thus, there is a ligand-induced internalization of the IR contributing to decrease insulin signalling [44,45]. Once insulin binds to its receptor, the β subunit of each $\alpha\beta$ complex autophosphorylates in Tyr residues. This leads to the binding and phosphorylation of the insulin receptor substrates (IRS) [45]. In addition to Tyr phosphorylation sites (activation), the IRS can be phosphorylated in serine/threonine (Ser/Thr) residues, acting as inhibitory feedback mechanism [46]. The phosphorylation in Ser/Thr is induced by insulin in hyperinsulinemic conditions, through the IRS1 serine phosphorylation by mTOR [47,48], dyslipidemia and inflammation. The pro-inflammatory cytokines released by macrophages can activate serine kinases including the inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β), c-Jun N-terminal kinase (JNK) or mTOR-ribosomal protein S6 kinase (mTOR-S6K) [44].

¹ LTP is a laboratory electrophysiological strategy where the stimulation of presynaptic neuron induces an increased and sustained response, recorded postsynaptically. It has been widely used to study the molecular mechanisms of memory formation. LTP requires glutamate neurotransmission through AMPA and NMDA glutamate receptors.

² LTD is another form of synaptic plasticity involved in memory formation and consolidation.

1.1.2. Insulin receptor substrates

Up to date four different isoforms of IRS in mammals have been described: IRS1, IRS2, IRS3 and IRS4. The members of this family may differ in tissue expression, subcellular distribution, and their ability to involve different intracellular signalling cascades. Generally, the mechanism of action is identical to all of them, the IRS when phosphorylated in Tyr residues bind and activate Src homology 2 (SH2) domain containing proteins, e.g. PI3K, the SH2 protein tyrosine phosphatase (SHPTP-2). This binding initiates multiple downstream cascades responsible of insulin pleiotropic actions ^[49,50]. The most studied substrates are IRS1 and IRS2, which have similar function, but not always interchangeable. Meanwhile, IRS3 and IRS4 appear to have a redundant role in the insulin signalling cascade and some authors ensure that humans lack IRS3 gene, being only a pseudogene ^[51]. Although in cultured rat adipocytes, the overexpression of both substrates (IRS3 and IRS4) promotes an increase of the recruitment of GLUT4 to the cell surface ^[52], the disruption of IRS3 and IRS4 genes in mice do not lead to any alteration of the growth or glucose homeostasis ^[53,54].

The first insulin receptor substrate identified was the IRS1, thus its role in the insulin/IGF1 signalling cascade is well established, mostly in neoplastic growth ^[55,56], cell metabolism, and proliferation ^[57]. In mice, deletion of IRS1 gene promotes growth retardation ^[58] and insulin resistance ^[59]. These mice are smaller compared to control mice.

IRS2 was discovered to mediate IRS1/IGF1 signalling after IRS1 and it was first suggested to be an alternative to IRS1 and that both substrates were functionally interchangeable. The IRS2 shares structural similarities with IRS1, and the 43% of their amino acid sequences (Fig. 1.1) ^[49]. However, the amino acids sequence from 591 to 786 of the IRS2 is an exclusive region (kinase regulatory-loop binding domain -KRLB-) not found in IRS1 ^[60]. This unique domain is an important difference with IRS1, underlying signaling differences between them ^[61]. For example, both IRS1 and IRS2 activates PI3K but its p110 α subunit is preferentially regulated by IRS2 ^[62], and IRS1 is associated with 3-phosphoinositide-dependent protein kinase-1 (PDK1) meanwhile IRS2 with PKC β ^[63]. Moreover, in terms of subcellular localization, IRS2 is mainly localised in the cytosol whereas IRS1 can also be located into the nucleus ^[64-66]. Studies in IRS1 knockout mice have shown that IRS2 can compensate the IRS1 signalling deficiency in liver and pancreatic β -cells, but not in skeletal muscle, brown adipocytes or fibroblasts ^[67-70]. Thus, IRS2 and IRS1 interchangeable activity is tissue dependent.

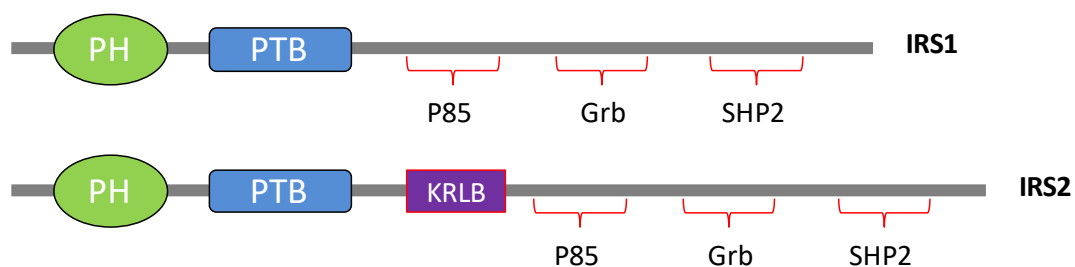


Fig. 1.1. IRS1 and IRS2 protein domains. Grb: growth factor receptor-binding protein; KRLB: kinase regulatory-loop binding domain; P85: regulatory subunit of phosphoinositide 3-kinase; PH: pleckstrin homology; PTB: phosphotyrosine binding; SHP-2: Src homology domain (SH)-containing phosphatase-2.

IRS1 and IRS2 are distributed throughout the brain; HPC, Hyp, prefrontal cortex, nucleus accumbens, caudate, putamen, ventral tegmental area or cerebellum; but in all these areas the IRS2 mRNA expression has been found higher than IRS1 expression ^[71]. Unlike these, the IRS4 is

mainly expressed in the Hyp, mostly in the arcuate nucleus and medial preoptic nucleus ^[72] (Fig. 1.2). IRS4 in Hyp plays an important role in feeding and energy balance ^[73], and the disruption of its gene is associated with central hypothyroidism ^[74].

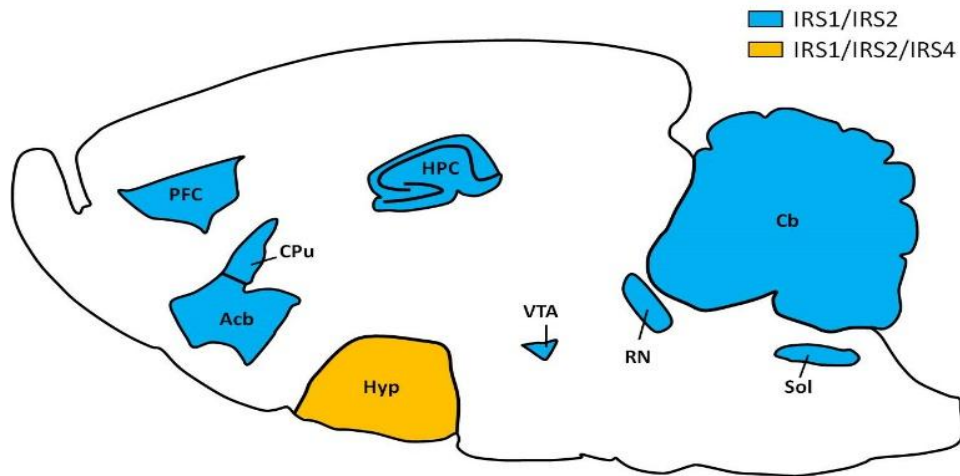


Fig. 1.2. Insulin receptor substrates (IRS) distribution throughout the mouse brain. Blue labelling indicates areas displaying IRS1 and IRS2 mRNA expression. Yellow labelling indicates areas displaying IRS1, IRS2 and IRS4 mRNA expression. Acb: nucleus accumbens; Cb: cerebellum; CPU: caudate putamen; HPC: hippocampus; Hyp: hypothalamus; PFC: prefrontal cortex; RN: raphe nucleus; Sol: solitary tract nucleus; VTA: ventral tegmental area. (Adapted from Kleinridders *et al.* 2014).

Alterations in IRS1 signalling affect brain function. In a post-mortem study of brains from patients affected by AD and tauopathies, IRS1 phosphorylation in 616 and 312 serines is increased compared to age-matched controls ^[75]. IRS1 pSer616 in the HPC of AD patients was strongly correlated with low episodic memory ^[76]. On the other hand, IRS1 pSer307 was found not associated to AD ^[77], whereas altered IRS1 pSer312 has been confirmed in plasma exosomes from AD patients ^[78]. Furthermore, the Baltimore longitudinal study of predictive biomarkers of AD has shown that, together with Tau, higher levels of IRS1 pSer312 in plasma neuronal enriched vesicles are best predictive factors for AD ^[79]. Interestingly, IRS1 may also mediate other neurotrophic factors signalling by binding to their receptors, e.g. TrkB (Tropomyosin receptor kinase B): the brain-derived neurotrophic factor (BDNF) receptor ^[80].

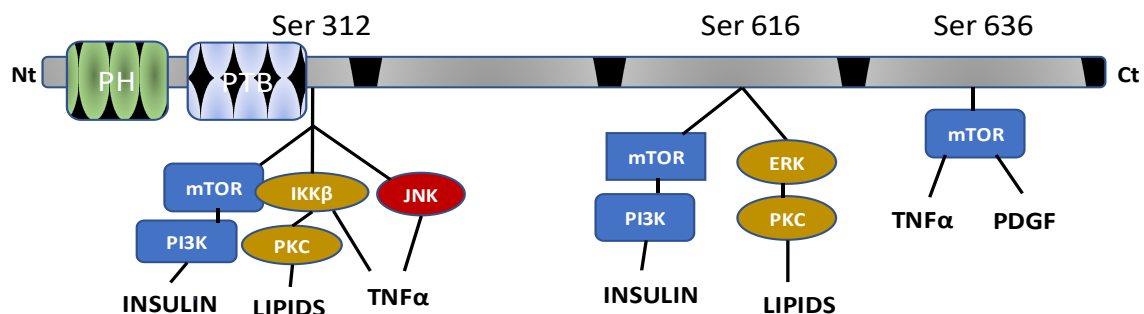


Fig. 1.3. Schematic representation of some serine phosphorylation sites in IRS1 and potential functions (in humans). The IRS1 phosphorylation in Ser312, Ser616 and Ser636 results in reduced insulin signalling (Adapted from Hançer *et al.* 2014).

Mice that have IRS2 gene disruption display reduced brain growth but normal somatic growth. Specifically, there is a 50% reduction of neuronal proliferation compared with wild type mice. Furthermore, knockout IRS2 mice show an increased Tau phosphorylation and an accumulation of Tau neurofibrillary tangles in the HPC^[81]. IRS2 is involved in memory processes, since disruption of IRS2 gene blocks the activation of postsynaptic NMDA receptors in the HPC, thus impairing LTP^[82].

All these data support the important role of insulin signalling for optimal brain function, showing that alterations on IR, IRS1 or IRS2 can cause feeding disorders, cognitive problems, and mood disorders.

1.2. Neuroinflammation and insulin resistance

Upon insult, the organism inflammatory response mediated by pro-inflammatory markers release (cytokines and chemokines) counteracts the damage. This response is a necessary physiological process to heal the tissue. However, if this response remains, it turns into a chronic state where there is a hyperactivation of tissue-resident macrophages that release pro-inflammatory cytokines continuously, inducing further damage. In fact, chronic inflammation underlies different human diseases such as neurodegenerative diseases (e.g. AD), cardiovascular diseases (e.g. atherosclerosis), autoimmune diseases, diabetes, mental disorders, cancer and even accelerated aging^[83]. Genetic and environmental events could increase the susceptibility to chronic inflammation. For example, rs1143634 polymorphism in interleukin (IL)-1 β (IL-1 β) gene is linked with periodontitis (teeth chronic inflammation)^[84]. Also, stressful life experiences increase the expression of pro-inflammatory cytokines, as has been reported in people suffering grief associated to familiar loss^[85].

The chronic inflammatory response that occurs in the CNS is called neuroinflammation^[86]. It is a process regulated by brain-resident macrophages, the microglia cells, required to recognize and eliminate any toxic component in the CNS^[87]. Microglia has a high capacity for mobility, and they can switch between two different phenotypes, M1 (pro-inflammatory) and M2 (anti-inflammatory), characterized by a different morphology and cytokine profile. The M2 phenotype is the “resting” type that actively monitors the brain in healthy conditions^[88]. The switch to M1 begins with the recognition of the toxic insults that are classified into pathogen-associated molecular patterns (PAMPs) or the damage-associated molecular patterns (DAMPs). The receptors that recognized them are the pattern recognition receptors (PRRs). These include several families of receptors: the toll-like receptors (TLRs) both plasma and endosomal membrane; the cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLR); the intracellular retinoic acid-inducible gene-I-like receptors; and the transmembrane C-type lectin receptors^[89]. PAMPs and DAMPs range from bacterial wall components (LPS) and virus capsid proteins, to debris released by dying cells and amyloid-beta (A β) oligomers^[90]. The activation of this system by the so-called inflammasome, initiates the inflammatory cascade, which results in the secretion of several pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), interferon gamma (IFN- γ) and IL-1 β , IL-6 and IL-18. The pro-inflammatory cytokines purpose is to orchestrate the neutralization and elimination of the toxic molecules. In normal conditions, once the toxic stimuli have been cleared, microglia switch to the M2 phenotype and secretes anti-inflammatory cytokines such as IL-4, IL-10 and IL-18, BDNF or nerve growth factor (NGF), whose role is to terminate the innate immune response and contribute to restore the synaptic function. However, under pathological conditions, microglia cells do not go back to their resting state, thus causing a chronic inflammation process, with the overproduction of pro-inflammatory cytokines and reduction of neuroprotective factors that in sustained situations become highly toxic, leading to neurodegeneration^[91].

Chronic neuroimmune system activation underlies the initiation and progression in many dementias, such as AD^[92-94]. In humans, increased levels of peripheral pro-inflammatory cytokines have been found in the serum of AD patients^[95]. Not only A β activates the microglia^[96], but also misfolded Tau interaction with microglia triggers inflammation^[97]. The elimination of the microglial receptor NLR family pyrin domain containing 3 (NLRP3) has shown to reduce brain A β levels in rodent models of AD^[98,99]; since then, the NLRP3 inflammasome has been deeply studied and characterized in AD^[100,101]. In addition to the neurological symptoms, neuroinflammation also underlies the psychiatric signs associated with AD, and for that reason, targeting neuroinflammation has also been proposed to treat those comorbid disturbances^[102].

Interestingly, different studies have shown that there is a lower incidence of AD amongst users of chronic non-steroidal anti-inflammatory drugs (NSAIDs)^[103,104], however NSAIDs administration do not counteract the cognitive decline once the disease is initiated^[105,106]. Other anti-inflammatory drugs such as Etarnercept (specific TNF α inhibitor), did not show any improve in cognition, behaviour or global function in a clinical trial with mild to severe AD patients^[107]. The failure of these treatments against inflammation to ameliorate AD symptoms indicates that

there may be other different factors involved, including insulin resistance. Thus, treating just inflammation may not be sufficient to effectively prevent/treat the disease ^[108,109].

Insulin metabolism alterations, especially the insulin resistance, are associated with metabolic and neurological diseases. Insulin resistance occurs when there is a reduced response to insulin, and it may be caused by different conditions, reduced levels of insulin/insulin-like growth factor-1 (IGF-1) receptor expression or binding and/or impaired IRS1/2 response. Peripherally, the principal role of insulin is to reduce blood glucose levels. Since centrally insulin is involved in many functions such as neuronal survival ^[110], synaptic function and maintenance ^[111], dendritic morphology and arbour development, energy homeostasis, learning and memory ^[112] (Fig. 1.4).

Several situations can bring about insulin resistance: metabolic syndrome caused by high caloric diet, sedentarism, obesity; genetic predisposition and neuroinflammation ^[113]. In several models of insulin resistance, increased Tau phosphorylation, and amyloid-beta precursor protein expression (APP) expression together with higher levels of A β oligomers and deposition has been found ^[114]. In addition, insulin resistance augments oxidative and ER stress, production of reactive oxygen species, mitochondrial dysfunction, and pro-inflammatory cascades ^[115]. Accordingly, T2DM is also an inflammatory condition since there is a sustained activation of macrophages and adipocytes with elevated pro-inflammatory cytokines release ^[116]. Since cytokines can cross the BBB, the peripheral increment of circulating pro-inflammatory cytokines can activate CNS cytokines production. Inflammatory markers in the Hyp has been found in subjects with high consumption of dietary fats ^[117,118]. Conversely, central insulin resistance alters the systemic glucose regulation by Hyp ^[119], worsening the metabolic syndrome.

Insulin resistance and inflammatory processes have been associated to classical aetiology processes of neurodegenerative diseases such as late-onset AD. In fact, AD has been often recognized as Type 3 diabetes ^[120]. Post-mortem analysis of AD patients brains, defects in insulin and IGF-1 signalling have been reported ^[121-123]. Specifically, deregulation of IRS1 and IRS2 is associated to insulin growth factor-1 receptor (IGF-1R) resistance ^[124].

For this reason, treatments targeting insulin resistance have been tested to fight AD progression. One of the strategies considered has been the administration of IN insulin. In a study using a streptozotocin-induced diabetic model, IN insulin restored learning and memory functions enhancing neurogenesis in HPC and olfactory bulb ^[125,126]. In a A β -inoculated rat model of AD, IN insulin reduced the level of tau hyperphosphorylation and microglial activation ^[127,128]. In humans, a systematic review on this strategy concluded that whereas IN insulin administration showed improvement in verbal memory and story recall, it was not effective on other aspects of cognition. Interestingly, the authors conclude that the treatment is affected by the apolipoprotein E (APOE) 4 isoform, where APOE 4 (-) patients displayed more benefits compared to APOE 4 (+) patients. This systematic review concluded that, although it is very safe (it does not interfere with systemic glucose levels), current data does not demonstrate that IN insulin can be used in humans as treatment for dementia of AD or mild cognitive impairment (MCI). Most importantly, these data supports that proper stratification by disease stage and APOE 4 carrier status must be considered to design better therapeutic strategies ^[129].

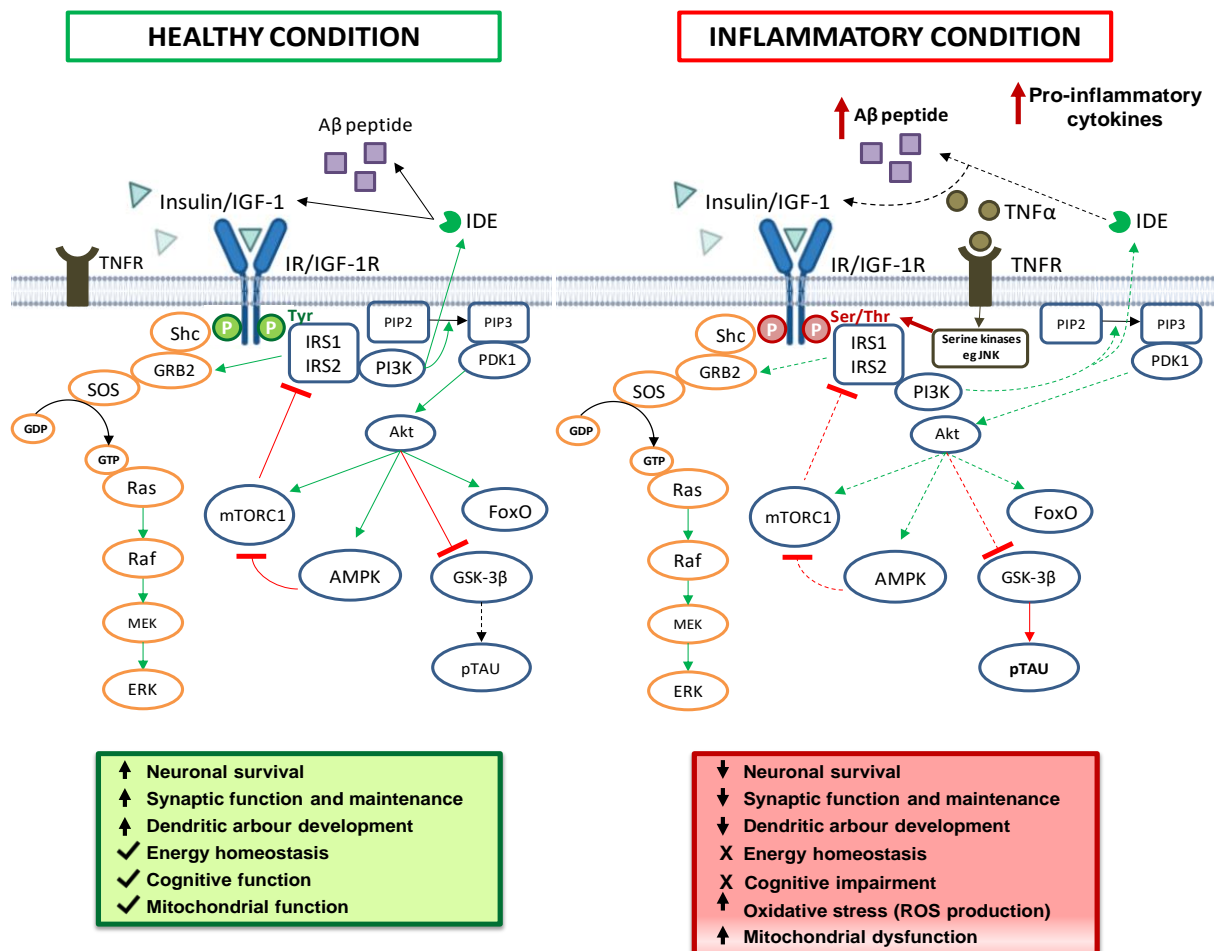


Fig. 1.4. Insulin signalling stimulates IRS1/2 tyrosine (Tyr) phosphorylation (healthy condition). Insulin resistance induced by IRS1/2 serine/threonine (Ser/Thr) phosphorylation (inflammatory condition). Upon ligand binding, recruitment of scaffolding proteins allows the stimulation of the Ras/ERK/1/2 and the PI3K/Akt pathways. In pathological situations, such as inflammation, insulin resistance reduces PI3K and Akt activity, leading to reduced IDE peptide degrading activity, and higher GSK-3 β activity. AMPK: adenosine monophosphate-activated protein kinase; A β : amyloid-beta peptide; ERK: extracellular signal-regulated kinase; FoxO: forkhead box O; GRB2: growth factor receptor-binding protein 2; GSK3 β : glycogen synthase 3-beta; IDE: insulin-degrading enzyme; IGF-1: insulin-like growth factor-1; IR/IGF-1R: insulin/IGF-1 receptor; IRS: insulin receptor substrate; JNK: c-Jun N-terminal kinase; MEK: mitogen-activated protein kinase; mTORC1: mammalian target of rapamycin complex 1; PDK1: 3-phosphoinositide-dependent protein kinase 1; PI3K: phosphoinositide 3 kinase; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; Ser/Thr: serine/threonine; Shc: src homology and collagen; SOS: son-of sevenless; pTAU: phosphorylated Tau; TNF α : tumor necrosis factor alpha; TNFR: tumor necrosis factor receptor; Tyr: tyrosine (Adapted from Bedse et al. 2015).

In addition, since hyperinsulinemia, associated to insulin resistance (serum levels >12 μ IU/mL) correlated positively with the severity of AD dementia^[130], other treatments designed to enhance insulin sensitivity, maybe a better choice of treatment, such as adenosine monophosphate-activated protein kinase (AMPK)- activators (e.g. Metformin^[131]). AMPK improves insulin signalling by inhibiting mTORC1 activity, which performs a negative feedback mechanism over IRS1 and IRS2 (Fig. 1.4). Interestingly, AMPK activity also displays an anti-inflammatory effect, decreasing proliferation of inflammatory cells and their adhesion to the blood vessel endothelium. AMPK activity also reduces amyloidogenesis, Tau hyperphosphorylation, and the activation of autophagic degradation^[132].

1.3. Abscisic acid: insulin sensitizer and anti-inflammatory nutraceutical

In 1963, two different laboratories specialized in plant biology described two substances: “dormin” involved in bud dormancy and growth inhibition^[133]; and “abscisin II”, involved in leaf abscission^[134]. Few years later, “dormin” and “abscisin II” were found to be the same chemical molecule and it was renamed as “abscisic acid” (ABA)^[135,136]. ABA is a phytohormone essential for the plant responses to biotic and abiotic stress situations (drought, extreme temperatures or high salinity)^[137].

Multiple ABA receptors have been identified in plants. One of these receptors is GCR2, a G-protein coupled receptor (GPCR). Interestingly, GCR2 amino-acid sequence displays a high homology to the mammals lanthionine synthetase component C (LANC)^[138,139]. In rat pancreatic beta cells and human granulocytes, ABA binds to the lanthionine synthetase component C-like protein 2 (LANCL2), a GPCR (G_i) (pertussis toxin-sensitive) that activates cyclic adenosine monophosphate (cAMP) production, leading to intracellular calcium increase and cyclic adenosine difosfato-ribose (cADPR) generation^[140]. In addition to LANCL2, ABA also interacts with the peroxisome proliferator-activated receptor γ (PPAR γ). This transcription factor is a nuclear receptor superfamily that support insulin responses in adipocytes^[141], and pancreatic beta cells^[142], likely through upregulation of GLUT4^[143] and IRS2 expression^[144]. The PPAR subfamily interacts with retinoid X receptors (RXRs) to create heterodimers. Interestingly, structural similarities, between ABA and retinoic acid (RA) have been identified^[145]. In addition to direct binding, ABA may also activate PPAR γ through cAMP/protein kinase A (PKA) signalling in macrophages and adipocyte tissue^[146], or via LANCL2 activation in monocytes^[147].

Interestingly, endogenous ABA production has been demonstrated in humans. Under elevated glucose concentrations pancreatic beta cells are activated and release ABA to stimulate insulin secretion using ADP as second messenger^[148], this signalling pathway has also been found in granulocytes^[149]. Furthermore, the release of endogenous ABA in response to glucose, is impaired in T2DM patients^[150].

The insulin-sensitizing properties of ABA had been demonstrated in T2DM animal models, high fat diet (HFD)^[118] or db/db mice^[151]. In humans this phytohormone enhanced glucose tolerance in T2DM patients^[152] and improved the postprandial glucose homeostasis in healthy subjects^[153]. The mechanisms underlying insulin-sensitizing properties could be via PPAR γ , similarly to other synthetic insulin sensitizer like the Thiazolidinediones (TZDs) family. Also via cAMP/PKA increase of glucagon-like peptide 1 (GLP1) release, which inhibits glucagon stimulation and raises insulin levels^[154], or by increasing glucose uptake by myocytes and adipocytes^[155].

In addition, ABA can regulate cell proliferation, however the results seem somewhat controversial, whereas in human mesenchymal stem cells^[156] and cultured hematopoietic progenitors^[157], ABA stimulates cell growth (via activation of NF κ B, the nuclear factor kappa-light-chain-enhancer of activated B cells); in glioblastoma cell culture, ABA induced apoptosis (via RA receptor α (RAR α)^[158]). Surprisingly, in these glioblastoma cells, ABA reduced the PPAR γ expression.

ABA can also regulate inflammatory mechanisms, but again, the results can be contradictory at least in cultured cells. On the one hand, ABA is pro-inflammatory, increasing monocyte chemoattractant protein-1 (MCP-1) levels to promote monocytes migration^[159]. On the other hand, the activation of PPAR γ can induce human monocytes into the M2 anti-inflammatory phenotype^[160]. Nevertheless, in *in vivo* studies point to an anti-inflammatory effect of ABA, in high-fat diet (HFD) animals, ABA administration ameliorated peripheral inflammation, reducing TNF α , adipocyte hypertrophy and macrophage infiltration in adipose tissue^[151].

About ABA central effects, before the publications from our group, there were only two studies, one preliminary data showing enhanced spatial memory in young rats treated with ABA^[161], and the effect of ABA reducing corticotrophin-releasing hormone (CRH) in rat Hyp^[162].

After our firsts publications (presented in this thesis) our group has shown that ABA can improve memory and reduce neuroinflammation in a triple transgenic mice model^[163] and very recently, this effect has been replicated in a 5x transgenic model of AD^[164] and in a streptozotocin rat model^[165]. Corroborating our initial studies and indicating that ABA may have a great therapeutic potential, suggesting an anti-inflammatory response, perhaps due to the insulin sensitizing properties.

Inflammation is a key factor in pain sensitization since it underlies pathologic pain conditions and promotes the development of hyperalgesia or allodynia (abnormal pain sensation to normal stimuli)^[166]. Recent data have demonstrated ABA effects on pain. Intrathecal administration of ABA increased pain threshold in male rats and this anti-nociceptive effect was mediated by PKA activation^[167]. Furthermore, ABA effects on pain sensitivity were completely abrogated when animals received intracerebroventricular naloxone (opioid receptors antagonist), thus the analgesic effect of the phytohormone could be carried out by opioid receptors activation^[168].

1.4. Brain networks: the memory circuitry

The major brain area involved in memory function is the HPC. Its role in memory formation was published with H.M case, the first patient which hippocampal ablation was reported. Due to an accident, H.M. began to suffer continuous epileptic attacks and so, the HPC was removed surgically to ameliorate the epilepsy. The epileptic attacks decreased but memory problems emerged. Specifically, H.M. displayed amnesia of declarative/explicit memory, which is the inability to remember events. Inside this kind of memory, H.M. had also damaged the episodic memory, so he could not recall particular personal experiences^[169]. In other words, the patient had retrograde amnesia, he could not remember personal events of the past, but also anterograde amnesia since he had impaired acquisition of new memories. However, the learning of motor skills, although unconscious, remained intact. Thus, the implicit memory function was not hurt after the hippocampal ablation. This was possible because there are more brain structures implicated. The surrounding cortical (e.g. perirhinal and entorhinal cortices) and non-cortical (e.g. septum, mammillary bodies) areas modulate hippocampal function and support different types of memory and processing.

Two cortical areas are particularly important for spatial memory: perirhinal and entorhinal cortices. Both areas send information to HPC, which is the responsible of the associative function by encoding information to a context^[170]. The perirhinal cortex (PC) is involved in visual recognition memory, processing specific details of objects (recollection) and identifying the ones explored previously (familiarity)^[171]. Specific parts of the hippocampal formation, the CA1 and subiculum, receive projections from PC, and CA1 send connections back to PC^[172] (Fig. 1.5). CA1-subiculum projections are critical for object-location learning and memory^[173].

The PC is also connected with entorhinal cortex (EC), an area involved in encoding spatial information. The medial part of EC (MEC) is responsible of spatial processing from an objective framework^[174], whereas the lateral part (LEC) represents the subjective/egocentric analysis of external items of the environment^[175]. In this area, specifically in MEC, a characteristic type of neurons called the grid cells are located. The firing of these neurons creates a grid-like regular hexagonal pattern that cover all the space available for the animal, providing a metric for the spatial environment^[176]. Grid cells relate to the place cells of the HPC. Place cells are CA1 pyramidal neurons that starts firing selectively when the animal is at one or few particular locations in the environment^[177]. Place cells not only indicate the position of the animal in a context but also the locations that the animal has visited previously^[178], so they play an important role in spatial memory. EC is the major cortical input of the dentate gyrus (DG) of the HPC to process spatial learning and memory, through the perforant pathway (pfp, Fig. 1.5).

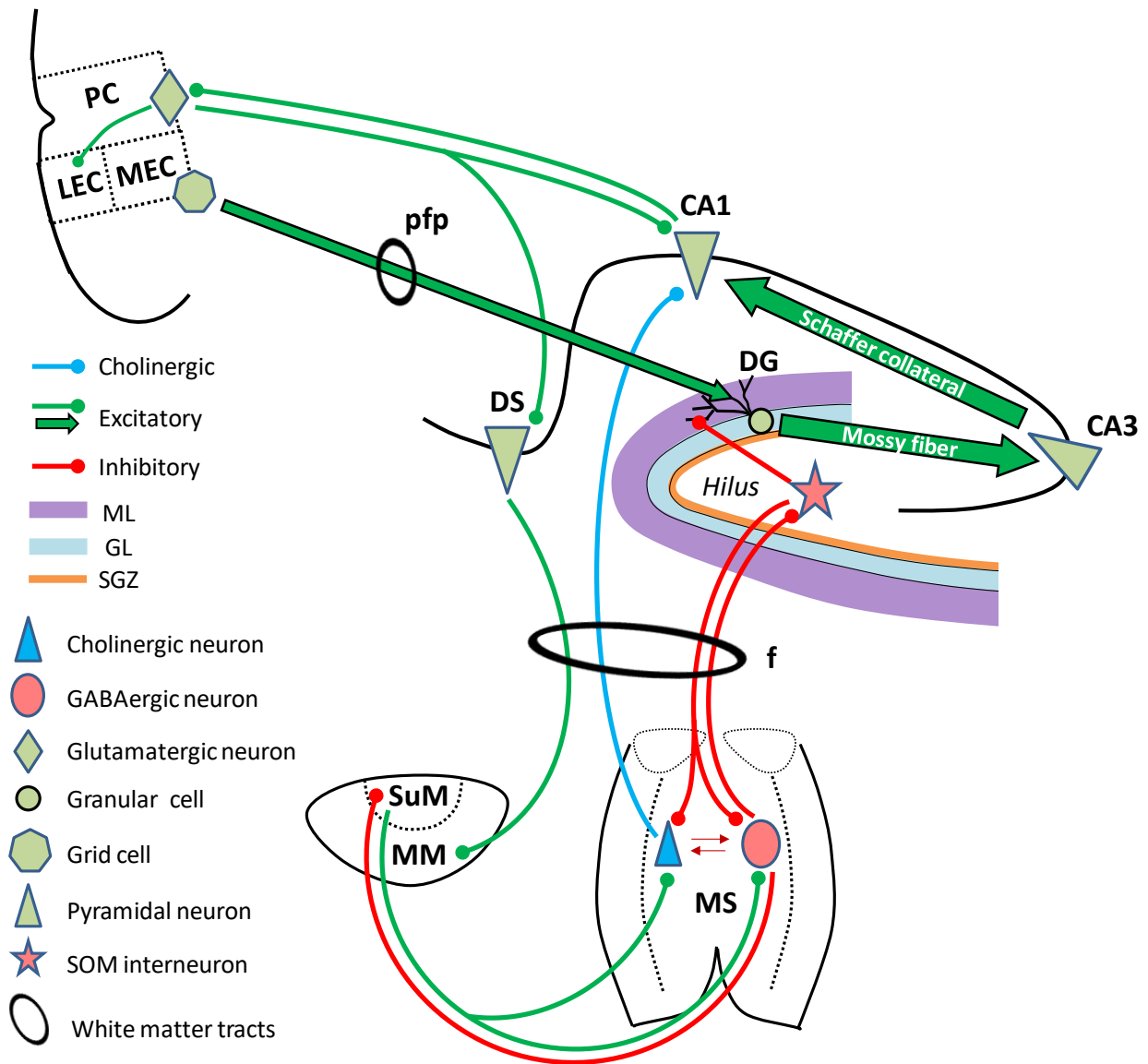


Fig. 1.5. Schematic hippocampal efferent/afferent connections.

Trisynaptic circuit: from the MEC (grid cells, green heptagon) to the granular cells in the DG, through perforant path, continuing to the CA3 pyramidal cells by the mossy fiber, and from these to CA1, via the Schaffer collateral. Regulation of this circuit by SOM interneurons (in the hilus, red star) is vital to determine CA1 firing pattern. **Septo-hippocampal connection.** Hilus interneurons send projections to GABAergic and cholinergic neurons in the MS through fornix. In turn, septal cholinergic and GABAergic neurons modulate CA1 and DG neurons, respectively. **Papez circuit.** The DS projects excitatory innervations to MM through the fornix, as part of the so-called Papez's circuit. Dorsal SuM sends glutamatergic projections and receives GABAergic feedback to and from the MS. In addition, the PC sends excitatory connections to the LEC, the DS and CA1, which in turn sends glutamatergic projections to PC. CA: Cornu Ammonis; DG: dentate gyrus; DS: dorsal subiculum; f: fornix; GL: granular layer; LEC: lateral entorhinal cortex; MEC: medial entorhinal cortex; ML: molecular layer; MM: mammillary bodies; MS: medial septum; PC: perirhinal cortex; pfp: perforant pathway; SGZ: subgranular zone; SOM: somatostatin interneuron; SuM: supramammillary nucleus (Adapted from Aimone et al. 2011, Khakpai et al. 2013, Vertes et al. 2001 and Yukie M. 2000).

The DG is a key point for generation of spatial memories. Information enters from EC to the granular layer (GL) of the DG. Granule cells are born by neurogenesis in the subgranular zone (SGZ) and communicate (via mossy fiber) to the CA3 pyramidal neurons, which, in turn, stimulate CA1 pyramidal cells (via Schaffer collateral) (green arrows in Fig. 1.5) ^[179]. This circuit is

extremely relevant in the formation of new memories, and GABAergic neurons have been demonstrated to play a decisive modulating role. Amongst them, PV and somatostatin (SOM) positive neurons from the *hilus* of DG. SOM neurons regulate both nearby PV interneurons and, via long projections through fornix also the ChAT and PV neurons in the medial septum (MS) ^[180] (Fig. 1.5). In turn, septal cholinergic and GABAergic neurons send ipsilateral projections through fornix to hippocampal formation to generate theta pattern, creating a regulatory feedback mechanism ^[181] (Fig. 1.5). Not only septo-hippocampal connections are involved in the regulation of memory formation, the MS is in synchrony with the supramammillary nucleus (SuM, dorsal to mammillary bodies -MM-) neurons ^[182] (Fig. 1.5).

In addition, the hippocampal formation is also an important part of the Papez's circuit: the major anatomical substrate of emotional control, mnemonic functions and episodic memories ^[183,184]. The information flows through different areas creating the following loop: subiculum of HPC → fornix → MM nucleus → anterior thalamic nucleus → cingulate cortex → parahippocampal gyrus (EC) → HPC ^[185].

In relation to human diseases like dementias, post-mortem studies of AD ^[186,187] and frontotemporal dementia ^[188] patients revealed severe atrophy of anterior thalamus, MM and HPC (areas involved in Papez's circuit). Moreover, the cholinergic hypothesis, had been considered for a long time in the aetiology of AD, as a consequence of septal cholinergic neurons degeneration ^[189]. Nowadays, this hypothesis is questioned as it cannot explain by itself the complex nature of the disease, however, cholinergic neurons death could be a consequence of a faulty circuitry (Fig. 1.5), for instance a lower inhibition from DG, since this would result in hyperexcitability. Supporting that idea, APOE 4 (the major genetic risk factor of late-onset AD) has been shown to induce hilar GABAergic alterations and subsequent memory dysfunction ^[190]. From that early report, in the last years, GABAergic cell therapy has been proposed as potential target for several neurological diseases ^[191] included AD ^[192,193].

2

HYPOTHESIS & OBJECTIVES

Hypothesis 1: The administration of abscisic acid (ABA) improves neuroinflammation and cognitive effects induced by high fat diet through its modulation of insulin resistance and inflammatory response.

Objective 1: Confirm that ABA administration ameliorates peripheral high fat diet-induced insulin resistance.

Objective 2: Study the effect of ABA administration on the cognitive impairment induced by high fat diet.

Objective 3: Evaluate the impact of ABA administration over inflammatory markers in hypothalamus and hippocampus.

Objective 4: Study the effect of ABA administration on the expression of insulin receptor substrate 1 (IRS1) and substrate 2 (IRS2).

Objective 5: Confirm the impact of high fat diet in hippocampal neurogenesis and if ABA administration counteracts that effect.

Hypothesis 2: The specific downregulation of insulin receptor substrate 1 (IRS1) in neurons of the dorsal hippocampus induces cognitive impairment in the absence of neuroinflammation.

Objective 1: Determine the effect of shRNA IRS1 AAV-derived particle injection in dorsal hippocampus over anxiety, spatial memory, and depressive-like symptoms.

Objective 2: Study the distribution of AAV-labelled dorsal hippocampus efferent connections.

Objective 3: Characterize shRNA IRS1 AAV-infected neurons in dorsal hippocampus.

Objective 4: Study the impact of shRNA IRS1 AAV particle injection in dorsal hippocampus over synaptic plasticity in hippocampal-labelled projections.

Hypothesis 3: The administration of ABA ameliorates behavioural symptoms and altered pain sensitivity in a preclinical model of attention deficit hyperactivity disorder (ADHD).

Objective 1: Establish the animal model of ADHD: neonatal 6-hydroxydopamine (6-OHDA)-induced lesion to confirm behavioural parameters.

Objective 2: Study the effect of ABA administration on the ADHD symptoms and pain sensitization developed by 6-OHDA-induced lesion.

3

Chapter 1

THE EFFECT OF ABSCISIC ACID CHRONIC
TREATMENT ON NEUROINFLAMMATORY
MARKERS AND MEMORY IN A RAT MODEL OF
HIGH-FAT DIET INDUCED
NEUROINFLAMMATION

3.1. Introduction

Obesity, a leading cause of type 2 diabetes^[194], correlates with cognitive impairment. Insulin sensitizers have been proposed as a promising tool for the reduction of obesity-induced insulin resistance and inflammation processes. The TZDs are a family of synthetic insulin sensitizer molecules; however, some of them have undesirable side effects^[195–197]. Thus, alternative compounds with analogous properties but fewer side effects are needed. The phytohormone abscisic acid (ABA) was found in mammalian cells more than 25 years ago^[198]. Since then, several studies have proposed it as a universal signaling molecule^[199,200]. Structurally, ABA is very similar to the TZDs. Indeed, ABA can improve glucose tolerance^[152], reduce the level of TNF α , and decrease adipocyte cell size in an *in vivo* model of obesity induced by HFD^[201]. Moreover, in human and murine pancreatic cell lines (RIN-m and INS-2 cells), ABA can increase glucose-stimulated insulin secretion^[202]. This effect can be repressed using pertussis toxin and PKA inhibitors^[148]. Dietary ABA also stimulates granulocyte function and macrophage infiltration in the adipose tissue^[203]. In mammalian cells, the LANCL2 shows high homology with the ABA receptor in plants, the Arabidopsis GCR2. Silencing the expression of endogenous LANCL2 in granulocyte cells can abrogate ABA induction of Ca²⁺ responses, whereas overexpression of LANCL2 enhances the ABA-mediated effects^[140]. Because of its role in the mediation of ABA effects, LANCL2 has been proposed as a therapeutic target for the treatment of inflammatory diseases and diabetes^[204].

Furthermore, ABA shows some molecular structural similarities to RA. The RA has beneficial cognitive effects, rectifying memory deficits in rodent models of AD. However, the clinical and animal model data show an association between RA administration and the symptoms of depression^[205]. Chronic treatment with ABA has beneficial antidepressant effects demonstrated by increased sucrose intake, increased swimming in the forced swim test, and reduced expression of CRH and RAR α mRNA in the rat hypothalamus in control rats, with no reported side effects^[162]. Moreover, preliminary data show an improved spatial memory in rats treated with ABA^[161]. Based on these data, we hypothesized that as ABA can modulate peripheral insulin resistance and immune response, it might also exert similar action centrally. Thus, in this work aims to show whether dietary ABA could restore cognitive deficits resulting from a HFD-induced neuroinflammation. HFD elevates the levels of neuroinflammation markers in the brain^[206] and it might constitute a link between obesity and degenerative disorders via insulin resistance^[207]. Furthermore, HFD has been shown to induce memory loss through elevation of inflammatory markers in HPC^[208].

We administered ABA (20 mg/L) in the drinking water to rats on either HFD or standard diet (SD). After eleven weeks of treatment, we compared the behavior of the four groups using two memory paradigms: the novel object recognition (NOR) and the T-maze. In addition, we measured ABA levels in the blood and cerebellum of all four groups using high-performance liquid chromatography (HPLC). We analyzed the microglia proliferation using immunohistochemistry. We demonstrated that the ABA administered in drinking water improved glucose tolerance and cognitive performance and decreased the levels of inflammatory markers in the hypothalamic areas. Our results confirm a therapeutic potential of this phytohormone in the peripheral metabolic alterations. The data also strongly suggest the potential beneficial effects of ABA in disorders of neuroinflammatory etiology, which has not been demonstrated before.

3.2. Methods

The aim of the experiment is to evaluate the central effects of the phytohormone, ABA in a model of neuroinflammation elicited by HFD, during 12-week treatment. Behavioral tests started on the 11th week, and sacrifices were carried out in the 12th week of ABA and diet treatment (Fig. 3.1).

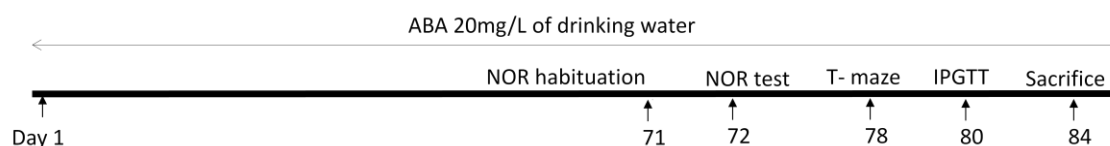


Fig. 3.1. Experiment design. Behavioral tests started in the 11th week, and the animals were sacrificed in the 11th and 12th week.

Animals and diet

Eight-week-old male Wistar rats were obtained from the Janvier Labs (Saint-Berthevin, France) and kept at the animal facility of the University Jaume I. The procedures followed the directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes. The experiments were approved by the Ethics Committee of the University Jaume I. The animals were maintained on a 12 h:12 h light–dark cycle and housed in pairs to reduce stress due to social isolation. Rats were divided randomly into four experimental groups: SD, control animals fed the standard rodent diet (Ssniff, Soest, Germany); SD-ABA, animals fed standard diet supplemented with ABA (Fernandez-Rapado, Spain) in the drinking water (20 mg/L); HFD, animals fed a high-fat diet (5736 kcal/kg, Ssniff) (Table 3.1); and HFD-ABA, animals fed high fat diet and ABA in drinking water (20 mg/L). We based our estimations in previous papers where ABA had been administered in the food pellet. Considering the daily food (20 g/day) and water average intake (50 mL/day), we estimated the amount of ABA per mL of drinking water, in order to doses in the same range as previous papers (100 mg/Kg)^[151]. In Guri study, the period of ABA treatment was shorter (36 days) than our study (12 weeks), therefore we considered that the ABA administration could be lowered (1 mg/day) to achieve optimal chronic effects. The four groups were fed *ad libitum* for 12 weeks. The HFD induces inflammatory effects in the peripheral tissues^[151] and the brain^[209]. The body weight and food and water intake were monitored twice a week per cage.

Table 3.1. Composition of high fat diet.

Crude Nutrients	%	Additives	per kg
Crude protein	24.4	Vitamin A (IU)	15000
Crude fat	34.6	Vitamin D ₃ (IU)	1500
Crude fibre	6.0	Vitamin E (mg)	150
Crude ash	5.5	Vitamin K ₃ (mg)	20
Starch	0.1	Vitamin C (mg)	30
Sugar	9.4	Copper (mg)	12
Fat	60 kJ%		
Energy	21.6 MJ (or kcal) ME/kg		

Novel object recognition (NOR)

Training chamber consisted of a black wooden box (81 x 61 x 52.3 cm). Rats were habituated to the testing environment for 30 min on two consecutive days. On the first day, the rats spent a 10-min habituation period in the experimental box, without objects. On the second day, each rat was placed in the box for the familiarization phase and allowed to explore two identical objects (heavy metallic jars). One group (easy task group) was left for 10 min, and the second group (difficult task group) was left to explore for 3 min. All the rats were then returned to their home cages for a 1-h intertrial interval. The box and objects were cleaned with a 30 % ethanol solution. In the test phase, the rats were returned to the box and allowed to explore a familiar and novel object for further 5 min (easy task group) or 3 min (difficult task group) ^[210]. Familiar and novel objects were alternated between left and right to prevent location predisposition. Both trial and test phases were recorded using a video tracking system (Smart 2.5.19, Panlab, Barcelona, Spain) for subsequent behavioral analysis. Exploration was defined as time spent sniffing within 1-4 cm of the object or touching it, always with the head oriented towards the object. Climbing over the object or running around it was not considered exploration.

T-maze

The T-maze was a three-arm maze; one arm (119.3 x 18.2 cm) was longer than the other two, which were identical (21.1 x 34.5 cm); the entire maze was placed above the floor. The longer arm was chosen as the start arm. The rats were habituated to the behavior room for 30 min. On the test day, the animals were allowed to explore the maze for 5 min, with access to two of the three arms (the home or start arm and the familiar arm). The rat was then returned to its home cage for a 2-h intertrial interval (difficult task group) or 90-min intertrial (easy task group), during which the maze was cleaned with 30 % ethanol. The rat was then placed back in the maze; this time the animal had access to all arms for 5 min ^[211]. The number of entries to the novel arm and the time rat spent in each arm was recorded using a video tracking system (Smart 2.5.19).

Glucose tolerance test

Rats were fasted overnight, and a drop of blood was taken from the tail before (t = 0) and 30 min (t = 30) and 120 min (t = 120) after glucose administration (2 g/kg; IP -intraperitoneal-). Plasma glucose was measured using Glucomen LX Plus glucometer.

HPLC ABA measurements

ABA was analyzed in all four groups using LC/ESIMSMS, essentially as described in ^[212], with slight modifications. Briefly, 1 g of frozen tissue was extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany) after spiking with 100 ng of d₆-ABA. After extraction and centrifugation, pH of the supernatant was adjusted to 3.0, and it was partitioned twice against diethyl-ether (Panreac, Barcelona, Spain). The organic layers were combined and evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was then resuspended in water-methanol (9:1) solution, filtered and injected into a UPLC™ Acquity system (Waters, Milford, MA, USA). The analyte was then separated on a reversed phase UPLC C18 column (Nucleodur C18, 1.8 μm, 50 x 2.0 mm, Macherey-Nagel, Barcelona, Spain). The solvents were methanol and water supplemented with 0.01 % acetic acid, applied at a flow rate of 300 μL min⁻¹. ABA was quantified with a Quattro LC triple quadrupole mass spectrometer (Micromass, Waters, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Quantitation of this hormone was achieved by external calibration with known amounts of standards.

Immunocytochemistry (ICC)

Rats were anesthetized with an overdose of pentobarbital (120 mg/kg Eutanax, Fatro, Barcelona, Spain) and transcardially perfused with saline (0.9 %) followed by 4 % paraformaldehyde (PFA) fixative in 0.1 M phosphate buffer, pH 7.4. After the perfusion, the brains were removed from the skulls and postfixed overnight at 4°C in PFA. Then, the brains were

immersed in 30 % sucrose for 48 h for cryoprotection. Sliding Microtome Leica SM2010R (Leica Microsystems, Heidelberg, Germany) was used to obtain 40- μ m-thick coronal frozen sections. The brains were cut in rostrocaudal direction; six series of slices were collected from each brain and stored at -20°C for analysis.

For ionized calcium-binding adaptor molecule 1 (Iba1) staining, we used a goat anti-Iba1 (Abcam, Cambridge, UK). Briefly, free-floating sections were rinsed twice in 0.05 M Tris-buffered saline (TBS, pH 8.0) and once in TBS with 0.2 % Triton X-100, at room temperature (RT). Sections were incubated in 4 % normal donkey serum for 1 h to reduce nonspecific labeling. Afterward, the sections were incubated in the primary antibody solution diluted 1:500 in 0.01 M phosphate buffered saline (PBS) containing 2 % normal donkey serum, TBS with 0.2 % Triton X-100, and 2 % bovine serum albumin for 24 h at RT. After washing off the excess of the primary antibody, the sections were incubated in biotinylated donkey anti-goat secondary antibody (Jackson) (1:200 in TBS, 0.2 % Triton X-100). Two hours later, the sections were transferred to the avidin-biotin-horseradish peroxidase complex solution (Standard Elite ABC kit, Vector Laboratories Burlingame, CA USA) for 90 min, followed by two rinses with TBS with 0.2 % Triton X-100, and two more with 0.05 M Tris/HCl pH 8.0. Then, the sections were processed in 0.05 M Tris/HCl pH 8.0 containing 3.125 mg of 3,3'-diaminobenzidine (DAB) and 2 μ L of H₂O₂ for 15-20 min. The reaction was stopped by adding an excess of 0.05 M Tris/HCl pH 8.0, followed by several rinses in PBS. Finally, the sections were mounted onto gelatin-coated slides, air-dried, dehydrated in alcohol, cleared in xylene, and coverslipped with dibutylphthalate polystyrene xylene (DPX) mounting medium.

RNA extraction and RT-PCR

Total RNA was extracted from the Hyp (n = 4-6 per group) using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The RNA samples were resuspended in 50 μ L of nuclease-free water. RNA concentration and quantification of total RNA was performed using Thermo Scientific NanoDrop 2000c, with the OD260/OD280. Genomic DNA was removed using DNase I, RNase-free (Life Technologies, USA), for 30 min at 37°C. The reaction was stopped by addition of 1 μ L of EDTA for 10 min at 65°C. The first strand cDNA was synthesized using the Prime-Script™ RT Reagent Kit (Perfect Real Time) (Takara Bio Inc., Shiga Japan). For each reaction, 1 μ g of RNA was used for reverse transcription, in a mixture of 4 μ L of 5x PrimeScript Buffer; 1 μ L of PrimeScript RT, 1 μ L of Oligo dT Primer (50 μ M), and 1 μ L of random primer (100 μ M). Enzyme mix was adjusted to a final volume of 20 μ L at RT. The mixture was incubated at 37°C for 15 min and heated at 85°C for 15 min to terminate the reaction. The cDNA was subsequently stored at -20°C. RT-PCR was performed in a volume of 10 μ L with 5 μ L of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Applied Biosystems Life Technologies, Carlsbad, CA, USA), 1 μ L of primer and 1 μ L of cDNA. All PCR reactions were performed under the following conditions: initial cycle at 98°C for 10 min followed by 40 cycles at 98°C for 10 s, 60°C for 10 s, and 72°C for 20s. Gene expression in the Hyp and HPC was quantified using a StepOnePlus Real-Time PCR system (Applied Biosystems Life Technologies). The RT-PCR primers for TNF α were Forward 5'GACCCTCACACTCAGATCATCTTCT3' and reverse 5'TGCTACGACGTGGGCTACG3'. Each sample was tested in triplicate. Data were analyzed using the comparative critical threshold method, with the amount of target gene normalized to the housekeeping gene β -actin. Relative gene expression was calculated using $2^{-\Delta\Delta C_t}$ relative to control.

3.3. Results

3.3.1. ABA treatment improves glucose tolerance of HFD-fed animals

Animals were weighed, and water and food consumption was monitored twice a week. Behavioral tests started on the 11th week, and sacrifices were carried out in the 12th week of ABA and diet treatment (Fig. 3.1). As expected, overnutrition affected the body weight, but ABA administration did not affect the weight gain in either group. The results are presented as the means \pm SE (n = 16 per group). Control animals fed SD increased their body weight from 444 ± 11.7 g (week 1) to 585 ± 12.0 g (week 10). Similarly, animals on SD supplemented with ABA increased their body weight from 446 ± 10.7 g (week 1) to 593 ± 10.2 g (week 10). This represents an increment of 132 ± 2.4 % and 133 ± 1.5 %, for SD and SD with ABA, respectively. However, animals fed HFD and HFD-ABA increased their body weight from 448 ± 11.7 and 453 ± 12.2 g (week 1) to 659 ± 16.0 and 669 ± 16.2 g (week 10), an increase of 148 ± 3.2 % and 148 ± 2.8 %, respectively (Fig. 3.2A). Data were analyzed using a two-way analysis of variance (ANOVA); time ($F_{(10,600)} = 994$, $p < 0.0001$) and diet ($F_{(3,60)} = 6.08$, $p = 0.001$) had a significant effect. Food and water consumption was measured per cage. Food intake data are represented by the mean (g) of food consumed \pm SE per cage each week (n = 8 per group). Weekly consumption was steady during the 12 weeks treatment, but the diet clearly affected food intake. The data were analyzed using two-way ANOVA ($F_{(3,28)} = 75.36$, $p < 0.0001$). To determine the ABA intake, we monitored weekly water consumption (Fig. 3.2C). Based on this information, we calculated ABA intake for both HFD and SD fed animals, and confirmed that both groups had a similar average weekly intake of the hormone, 12.30 ± 1.4 and 13.27 ± 0.8 mg/week/cage, respectively (Fig. 3.2D).

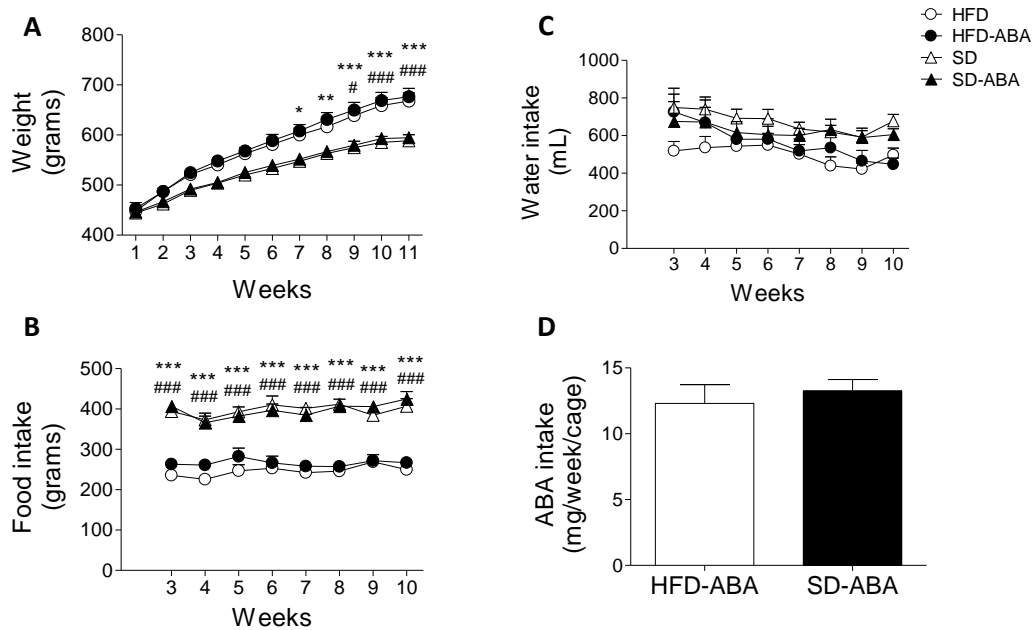


Fig. 3.2. Body weight in grams (A), food intake (B), water intake (C) and ABA weekly consumption per cage (D) of rats fed high-fat diet (HFD, white circles); HFD with ABA (HFD-ABA, black circles); standard diet (SD, white triangles), and SD-ABA (black triangles) for 10–11 weeks; n = 16 per group. Values are mean \pm SEM. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ for HFD vs. SD. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ for HFD-ABA vs. SD.

It has been reported that dietary ABA given in the food pellets (100 mg/kg food) can improve glucose tolerance^[151]. To confirm that, in our model, ABA (20 mg/L of drinking water) improved the glucose homeostasis, we performed a glucose tolerance test by intraperitoneal injection of glucose (2 mg/kg) in fasted animals. After 11 weeks of treatment, the animals fasted overnight (12–13 h). The basal glucose levels were similar in all groups; 30 min after glucose injection, blood glucose levels increased. No significant differences were observed between the

groups. However, two hours after injection, glucose levels in HFD group remained higher (235.4 ± 36 mg/dL) than in HFD-ABA group (158.9 ± 11.23 mg/dL). In the latter group, the glucose levels were similar to those in control groups, SD (143.9 ± 10.24 mg/dL) and SD-ABA (152.6 ± 15.52 mg/dL). The data were analyzed using one-way ANOVA, with Newman–Keuls post-hoc test ($p < 0.05$) (Fig. 3.3).

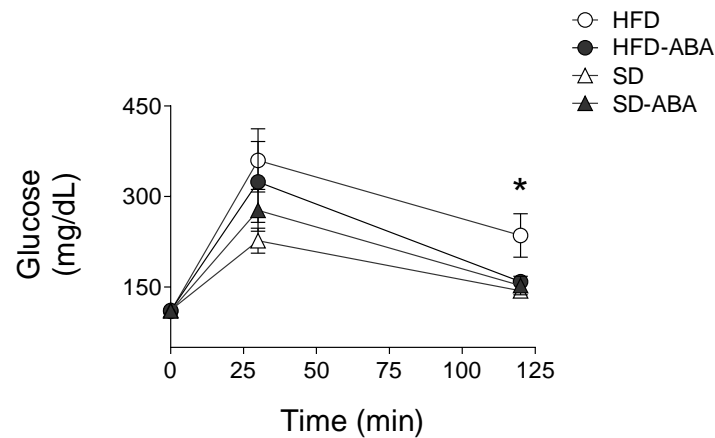


Fig. 3.3. The effect of dietary ABA (20 mg/L drinking water) on blood glucose levels in response to intraperitoneal (IP) glucose tolerance test. After 12 weeks of treatment, animals from the four groups were fasted overnight (12–13 h) and given 2 mg/kg of glucose by IP injection. Blood drops were taken from the tail, and plasma glucose concentration measured at the indicated times. Data are presented as the mean \pm SEM. White circles represent rats that fed HFD; black circles, HFD-ABA; white triangles, SD; and black triangles, SD-ABA. Data points at 120 min were analyzed using one-way ANOVA followed by post-hoc Newman–Keuls test. *, $p < 0.05$ vs. HFD.

3.3.2. ABA restores memory impairment induced by HFD

HFD can induce memory impairments in rodents^[213] and humans^[214]. To examine the effect of chronic ABA administration on cognitive performance, the animals were subjected to two behavioral paradigms that evaluate the memory in rodents, NOR and T-maze test. NOR exploits the innate exploratory preference of novel objects exhibited by rodents. This paradigm examines the capability of the animal to remember a familiar object when presented with a new one. We observed no significant differences in the time spent exploring the identical objects during familiarization phase (Fig. 3.4A and C). During the test, all four groups spent much more time exploring the novel object than the familiar one, and neither the diet nor ABA treatment changed these parameters (Fig. 3.4B). However, we observed differences when both the familiarization time and test time were reduced to 3 min. In the HFD group, the times for familiar and novel object did not differ significantly, suggesting impairment in remembering the familiar object. HFD-ABA animals behaved in the same way as the control groups (SD and SD-ABA), indicating that ABA could abrogate the HFD-induced impairment (Fig. 3.4D).

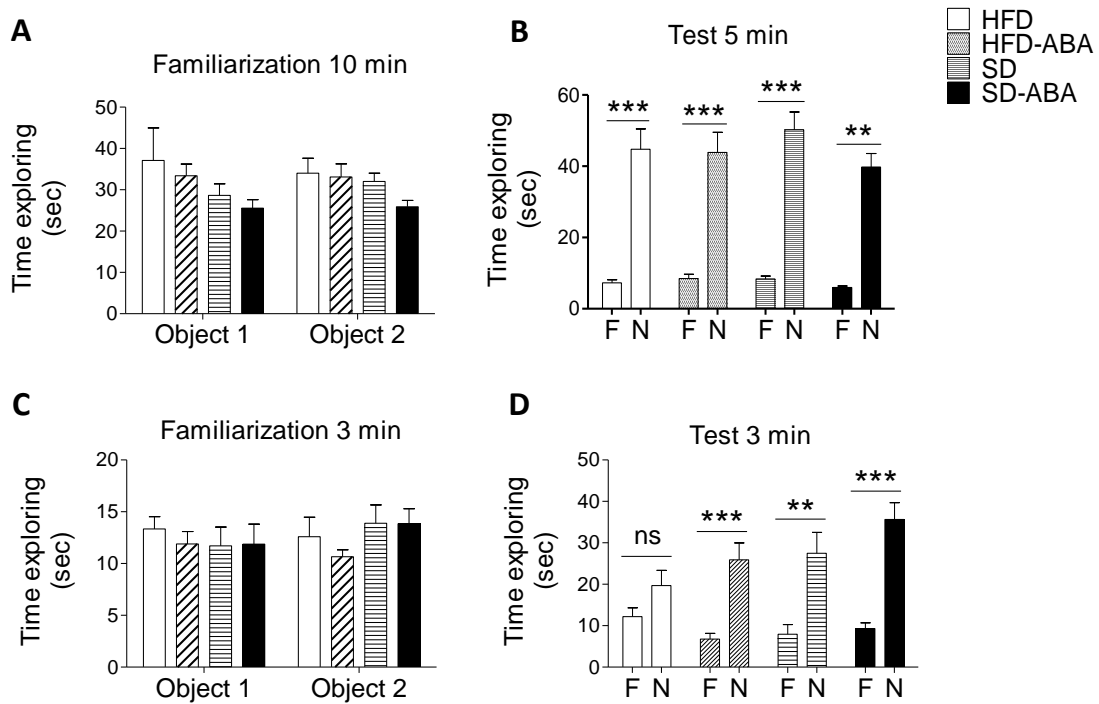


Fig. 3.4. Behavioral data for rats on a standard diet (horizontal lines, SD), high-fat diet (white columns, HFD), and supplemented with ABA in drinking water (20 mg/mL), HFD-ABA (diagonal lines) and SD-ABA (black columns) ($n = 8$). Novel Object Recognition data: time exploring two similar objects during familiarization phase (A, C) and time exploring familiar and novel object during test phase (B, D). Data are presented as mean \pm SEM and analyzed using paired Student *t*-test, familiar vs. novel. **, $p < 0.01$; ***, $p < 0.001$; ns, non-significant.

In the T-maze test, we recorded the number of entries to both maze arms, one of which was familiar and the other was novel because it had been closed during the habituation. We observed that rats fed SD, SD-ABA, and HFD-ABA had a significantly higher number of entries to the novel arm than to the familiar one. This was not the case for the animals fed HFD only, suggesting that this diet might impair the memory of the familiar arm (Fig. 3.5A). The data were analyzed using unpaired Student's *t*-test, comparing familiar and novel arm; * $p < 0.05 < **p < 0.01$. Interestingly, when the test was performed with a longer inter-trial time between familiarization and test phases, which may be a more difficult working memory task, HFD-ABA did not rescue the alternation behavior shown by the HFD group. We found that the difference between the number of entries to the two arms was no longer significant for HFD-ABA animals, in similarity with HFD group. Both SD and SD-ABA group maintained a significant difference in the exploratory behavior, entering the novel arm more often (Fig. 3.5B).

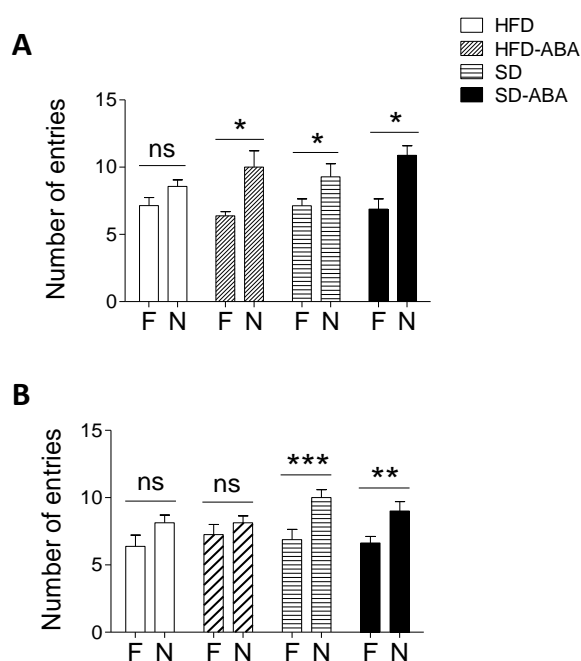


Fig. 3.5. Behavioral data for rats on a standard diet (horizontal lines, SD), high-fat diet (white columns, HFD), and supplemented with ABA in drinking water (20 mg/mL), HFD-ABA (diagonal lines) and SD-ABA (black columns) ($n = 8$). T-maze data: number of entries to novel and familiar arm after 90 min intertrial interval (A) and number of entries to novel and familiar arm after 2 h intertrial interval (B). Data are presented as mean \pm SEM ($n = 8$) and analyzed using paired Student t-test, familiar vs. novel. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, non-significant.

3.3.3. ABA administered in the drinking water can cross the BBB

ABA levels in the blood and brain (cerebellum) were measured using HPLC. We detected variable amounts of circulating ABA in treated animals. However, we were unable to detect ABA under the same conditions in untreated animals, confirming that we, indeed, were observing exogenous ABA (Table 3.2). It has been reported that after intraperitoneal injections, ABA is widely distributed in the brain^[161]. To ascertain that ABA crosses the BBB in our model, we measured the ABA levels in the cerebellum as a sentinel. In similarity with the serum examination, ABA was only detected in the brains of treated animals (Table 3.2). Interestingly, only a very small proportion of circulating ABA crosses the BBB. Thus, we confirmed that our method of ABA administration in drinking water was appropriate for measuring potential peripheral effects; the compound could cross the BBB and might be responsible for the observed behavioral changes.

Table 3.2. ABA quantification by HPLC. Cerebellum (A) and blood (B) were extracted from all four groups of treated animals.

Cerebellum		Serum	
Diet group	ABA detected (ng/gr tissue)	Diet group	ABA detected (ng/gr tissue)
HFD	N.D.	HFD	N.D.
HFD-ABA	1.510 \pm 0.030	HFD-ABA	196.8 \pm 82.57
SD	N.D.	SD	N.D.
SD-ABA	2.980 \pm 0.100	SD-ABA	47.95 \pm 13.80

N.D.: not detected

ABA was only detected in animals that had received exogenous administration of ABA in the drinking water (20 mg/L)

3.3.4. HFD intake increases TNF α and reactive microglia levels in hypothalamus. ABA can effectively counteract these changes

Next, we measured the levels of inflammatory markers in the brain. We used immunohistochemistry to detect the microglia-specific cytoplasmic marker Iba1^[215] in the Hyp (Fig. 3.6A). We observed that the number of microglial cells increased significantly in rat Hyp of HFD-fed rats (1613 ± 260.5 cells/mm²) in comparison with SD-fed controls (754 ± 135.2 cells/mm²). The ABA treatment reduced this effect in HFD animals (888 ± 158.8 cells/mm²) but did not affect SD-fed animals (794 ± 178.4 cells/mm²) (Fig. 3.6B; one-way ANOVA, $p = 0.0135$). Representative images of microglia in the Hyp are shown in Fig. 3.6 (HFD, Fig. 3.6C; HFD-ABA, Fig. 3.6D; SD, Fig. 3.6E; and SD-ABA, Fig. 3.6F). In HFD group, apart from the increase in the number of Iba1-positive cells, the microglia cells were less ramified and more amoeboid than in controls, indicating an activated state (insets in images). On the other hand, we found no significant differences in HPC, under the same conditions in all four groups (data not shown).

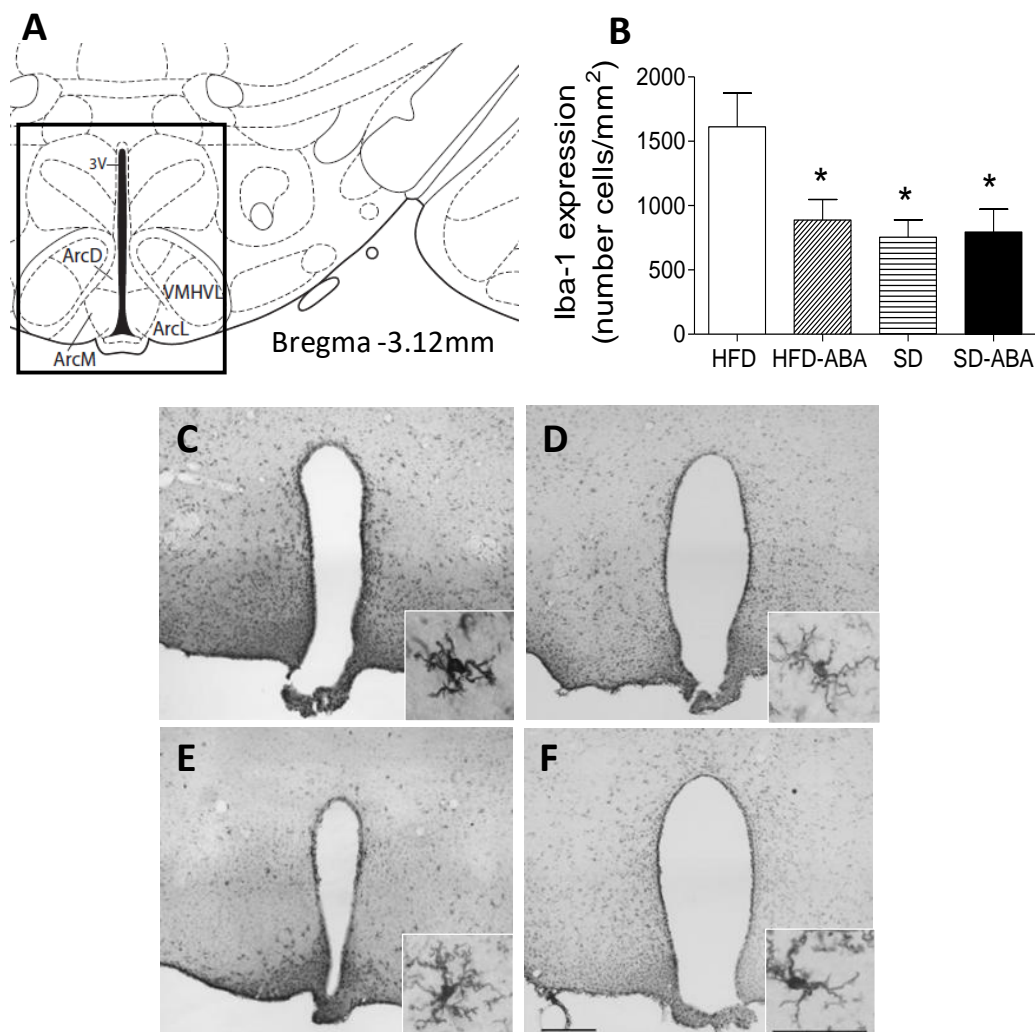


Fig. 3.6. Within the hypothalamus (Hyp), ABA curtails the increase in microglia staining induced by high-fat diet (HFD). Map showing the location of the analyzed hypothalamus (A). Anterior (bregma -1.80 mm) and medial (bregma -3.12 mm) hypothalamus were used for quantification (B). HFD fed rats (C); HFD-ABA (D); standard diet, SD (E); and SD-ABA (F). Calibration bar, $200 \mu\text{m}$ (C–F). Data are represented as the mean \pm SEM of density of cells per mm² ($n = 6$). Data were analyzed using one-way ANOVA followed by post hoc Newman–Keuls test; *, $p < 0.05$ vs. HFD. The reactive microglia are shown in the insets at high magnification. Calibration bar, $50 \mu\text{m}$.

Next, we examined the levels of inflammatory and anti-inflammatory cytokines in the Hyp. The Hyp was dissected from all groups, and total RNA was isolated. Quantitative PCR was carried out to determine the expression levels of different cytokines. We observed that TNF α levels increased in the Hyp of HFD-fed rats in comparison with controls, confirming the existing reports^[216,217]. ABA administration restored TNF α levels to control values (Fig. 3.7).

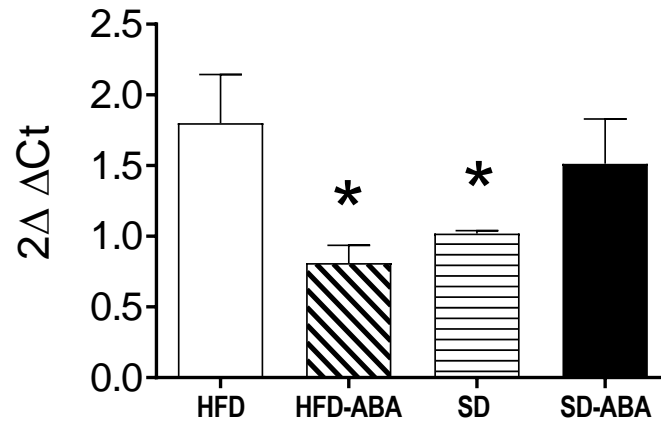


Fig. 3.7. Cytokine mRNAs levels in the hypothalamus: pro-inflammatory cytokine TNF α in rats fed high fat diet (HFD, white columns); HFD supplemented with ABA in drinking water (20 mg/L) (HFD-ABA, diagonal lines); standard diet (SD, horizontal lines); and SD supplemented with ABA (SD-ABA, black columns). Data are presented as mean \pm SEM (n=4–6 rats) and analyzed using one-way ANOVA followed by Newman–Keuls Multiple Comparison Test; *, $p < 0.05$ vs. HFD.

3.4. Discussion

Excessive energy intake is a major cause of obesity, which is recognized as one of the greatest threats to human health in the Western societies. Malfunction of hypothalamic neurons appears to underlie the pathology of metabolic diseases^[218]. Even though the relationship between obesity (excess body fat) and neurodegeneration is highly complex, many sources link obesity and high fat consumption with certain neurodegenerative processes^[207]. Growing evidence demonstrates that HFD induces insulin resistance, immunological and synaptic alterations in different brain areas^[219,220], and cognitive impairment^[221].

In animal obesity models, the phytohormone ABA, related structurally to insulin sensitizers (e.g. TZDs) and memory-improvement molecules (such as RA), ameliorates the symptoms of type 2 diabetes. It targets PPAR γ in a manner similar to the TZDs class of anti-diabetic drugs^[151,222]. In this study, we demonstrate, for the first time, that ABA can counteract some of the neurological effects (cognitive impairment and neuroinflammation markers) induced by HFD. We, in this study, and others have found that ABA can cross the BBB, but whether the improvement on cognitive functions is caused by direct action of ABA in brain, remains elusive.

Our protocol was designed to avoid an increase in behavioral anxiety. ABA was administered in the drinking water (20 mg/L) and not in food pellets since rats fed on an HFD tend to eat less than controls^[223]. The dose was based on previous studies reporting dietary administration of ABA^[151]. The vehicle used to dissolve ABA was added to water for SD and HFD groups to match any possible taste alterations. Some studies have used intraperitoneal injections (IP), which guarantee exact dosing but are highly anxiogenic. Another innovation in our study was housing the animals in pairs to reduce stress due to isolation^[224]. We considered these issues important since stress can affect feeding and cognitive behavior in many indirect ways^[225]. Therefore, reducing the stress levels (by avoiding isolation and daily injections) was desirable. To examine the intake of ABA, we examined the average weekly water consumption in groups SD-ABA and HFD-ABA. Daily ABA intake was approximately 1.5–1.8 mg/kg. During the 12-week period, the intake average was uniform in all groups. To validate our strategy as an effective way of drug administration, we carried out a glucose tolerance test in all four groups. We confirmed that ABA improves glucose tolerance, reducing blood glucose levels to the normoglycemic range two hours after glucose injection.

The effect of specific diets on cognitive performance in rodents has been investigated using different behavioral paradigms. Diets with increased sugar and fat content cause impairment in spatial and working memory^[226–228] in both rats and mice. The link between obesity and inflammation of central nervous system is widely accepted; the Hyp is the area where inflammatory markers are most evident. Neuroinflammation underlies cognitive impairment^[194]. On the other hand, some reports have suggested that ABA improves spatial memory^[162]. ABA has anti-inflammatory and insulin-sensitizing properties, and is a PPAR γ agonist^[229,230]. Some reports have shown that PPAR γ agonism in the central nervous system ameliorates cognitive function^[231,232]. Therefore, we hypothesized that ABA would alleviate the HFD-induced neuroinflammation and cognitive defects. To follow up this assumption, we examined the performance of the rats in two simple memory tests, NOR and T-maze. In contrast to other reports, ABA did not significantly affect the animals fed a control diet. This discrepancy might have been caused by differences in animal handling. As mentioned above, we housed the animals in pairs to reduce the isolation anxiety and ABA was administered in the drinking water, reducing the IP injection-induced stress. Under these conditions, the reduced anxiety levels might have a positive effect on test performance. In the studies showing antidepressant-like effects of ABA, the compound has been administered daily using an IP injection^[161]. We believe that in our model, the effect of ABA is independent of its antidepressant activity.

In a NOR working-memory test, all groups, including the HFD group, were able to discriminate between the novel and the familiar object. However, when the time spent in familiarization and test phases was shortened, thus reducing/hurting the acquisition phase, HFD-fed animals spent similar time exploring the novel and the familiar object. This indicated that they no

longer remembered the familiar object. This behavior was efficiently counteracted by ABA treatment. ABA-treated animals behaved like the members of other groups, spending significantly more time exploring the novel object.

The alternation test (the T-maze test) is considered a reliable test to evaluate spatial working memory in rodents^[233,234]. Using this paradigm, we found that HFD could impair alternation; the animals had difficulties in remembering the previously visited arms. Chronic treatment with ABA can restore this capability. However, when the intervals between the tests were increased, making the task harder, HFD-ABA-fed animals no longer alternated, behaving like the animals in the HFD group. This indicated that they had lost the ability to discriminate between the familiar and novel arms. We would not attribute these differences to ABA metabolism; not only ABA is administered chronically, but during the inter-trial interval rats have access to water and food. The peripheral anti-inflammatory effects ABA are well known. However, to the best of our knowledge, there is no data on the effect of ABA on the cerebral immune response *in vivo*. Moreover, *in vitro* data are controversial; some studies show that ABA activates the microglia^[235], whereas others report no effect^[236]. Among the different types of glia, the resident macrophages in the brain, microglia, are responsible for cell debris clearance and release of cytokines that recruit other immune-responsive cells to the central nervous system^[237]. In the metabolic syndrome induced by HFD, microglia in the Hyp adopts a pro-inflammatory state, which is linked to an abnormal increase in the production of pro-inflammatory cytokines. These might be toxic to the neurons^[238,239]. In this study, we investigated the microglial expression in the Hyp in the four study groups. We examined the immuno-reactivity of Iba1, a cell marker upregulated during microglial activation^[215]. In contrast to other studies of ABA effect on microglia *in vitro*, reporting either activation^[233] or no effect^[236], we demonstrated that *in vivo*, ABA reduces the number of Iba1-positive cells. This finding suggests an important practical application; the microglia hyperactivation has been found in a number of alterations including traumatic brain injury^[240], cerebrovascular accidents^[241], neurodegenerative disorders^[242,243], epilepsy^[244], schizophrenia^[245], and depression^[246]. In addition, we analyzed Iba1 expression in dorsal HPC, but HFD did not increase microglia proliferation compared to control groups (data not shown).

Further to test the anti-inflammatory effects of ABA in the central nervous system, we measured the hypothalamic and hippocampal cytokine levels. The activated microglia synthesize and secrete pro-inflammatory cytokines, such as TNF α ^[247]. We observed an increase in TNF α levels in the Hyp of HFD-fed rats. ABA treatment rescued this effect, lowering TNF α to control levels.

Importantly, in control rats, ABA treatment may worsen some parameters, that even not statistically significant, could point to a therapeutic window where ABA can restore deficits induced by HFD inflammatory processes, but it may not be advisable if there are not previous deficits. Further studies are required to elucidate the range for optimal ABA action.

In summary, we found that chronic treatment with ABA reduced HFD-induced microglia activation in Hyp, as revealed by Iba-1 staining. Also, TNF α levels were altered in Hyp of HFD-fed animals, and these changes were counteracted by ABA administration. Moreover, ABA ameliorated the HFD-induced impairments in cognitive performance. We found no changes in reactive microglia density in HPC. In this study, we show that hypothalamic inflammation can correlate with mild alterations in cognitive performance. Finally, based on our results, we conclude that ABA has a protective effect in neuroinflammation conditions, lowering microglia and TNF α levels and restoring cognitive function. Given the fact that ABA can cross the BBB; we postulate that this molecule could have a potential therapeutic value in the treatment of diseases of neuroinflammatory etiology.

4

Chapter 2

ABSCISIC ACID SUPPLEMENTATION RESCUES
HIGH FAT DIET-INDUCED ALTERATIONS IN
HIPPOCAMPAL INFLAMMATION AND IRSS
EXPRESSION

4.1. Introduction

Adult neurogenesis is a sensitive process that is very susceptible to different toxic insults, such as the presence of A β plaques (characteristic in AD) ^[248], insulin resistance ^[249], or inflammation ^[250]. Impairment of neurogenesis is associated with alterations of hippocampal-dependent functions, including spatial awareness, long-term memory, emotionality, and mood ^[251,252].

For more than 20 years, neuroinflammation has been considered to underlie the onset of neurodegenerative disorders ^[253], including AD ^[254–257]. Importantly, neuroinflammatory processes resulting from obesity and stress can induce insulin resistance ^[258]. These processes cause a considerable increase of reactive microglia, which secrete a variety of cytokines (e.g. TNF α), which activate specific intracellular cascades, including IKK β and JNK ^[259]. Aside from this, a large body of evidence has accumulated correlating insulin resistance induced by chronic inflammatory processes and the development of the disease ^[260]. In contrast, numerous preclinical and clinical studies have shown how insulin sensitization improved cognitive damage, cytokine levels, and mitochondrial function after a fat enriched-diet period ^[261,262].

Interestingly, insulin/IGF-1 signalling has been shown to protect against these toxic insults and improves neurogenesis ^[263]. In line with this, physical exercise improves insulin sensitivity and increases hippocampal neurogenesis ^[264,265]. The mechanisms of the exercise-induced improvement of neurogenesis may be a result of several factors, importantly the augmented blood flow to the brain ^[266] with the consequent affluence of growth factors, in particular IGF-1 ^[267]. Highlighting the importance of IGF-1, pilocarpine-induced epilepsy has been shown to increase the IGF-1 expression via cAMP response element-binding (CREB) protein stimulation in the SGZ-surrounding microglia, improving neurogenesis ^[268]. Another important factor in neurogenesis is the BDNF ^[269]. In fact, there is evidence that hippocampal BDNF expression is also augmented by exercise ^[270]. Furthermore, a crosstalk between BDNF and IGF-1 has been reported to play an important role in exercise-induced neurogenesis ^[271].

Insulin and IGF-1 signalling cascades are very similar: both peptides exert their actions through tyrosine kinase receptors via the subsequent activation of the IRSs ^[272–274]. Of the four known receptor substrate isoforms, the IRS1 and IRS2 isoforms are highly expressed in the brain ^[121,275]. Insulin and IGF-1 receptors are expressed in dentate gyrus in rat and mouse brains ^[276–279], but the specific expression of the IRS in these areas is largely unknown. Neuroinflammation may induce insulin resistance through IRS inactivation by specific serine phosphorylation. In humans, IRS1 P-Ser 307 may determine insulin resistance in type 2 diabetes ^[50,280]. One of the consequences of pSer in IRS1 is to increase the degradation of the protein using ubiquitination ^[47]. However, this process does not appear to occur for IRS2 degradation ^[281], which suggests differential regulation of both proteins, at least in certain cell types.

In addition to exercise, other factors, such as phytohormones have been proposed as neuroprotectors, precisely due to their insulin sensitizer activity and anti-inflammatory properties ^[282]. For instance, ABA is a PPAR γ agonist ^[147] that can improve glucose tolerance in obesity models, reduce neuroinflammation, and restore HFD-induced neurological alterations ^[118,152,161].

In this study, we evaluate the effects of HFD-induced neuroinflammation and ABA treatment in the expression levels of several members of the insulin/IGF-1 pathway, along with inflammation markers, in the HPC. One of the well-known effects of HFD exposure is the reduction of adult hippocampal neurogenesis ^[283]; therefore, we evaluated whether ABA treatment could rescue the HFD-induced reduction in hippocampal neurogenesis via its anti-inflammatory and insulin sensitizing properties.

4.2. Materials and Methods

Animals and Diet

Eight-week-old male Wistar rats (Janvier Labs, Saint-Berthevin, France) were kept at the animal facility of the University Jaume I. The procedures followed directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes. The experiments were approved by the Ethics Committee of the University Jaume I (approval number 2014/VSC/PEA00209). The animals were maintained on a 12 h:12 h light–dark cycle and housed in pairs to reduce stress due to social isolation. Rats were divided randomly into four experimental groups: SD, control animals fed the standard rodent diet (Ssniff, Soest, Germany); SD-ABA, animals fed standard diet supplemented with ABA (Fernandez-Rapado, Spain) in their drinking water (20 mg/L); HFD, animals fed an HFD (5736 kcal/kg, Ssniff), and HFD-ABA, animals fed an HFD and that had ABA in their drinking water (20 mg/L). We have used ABA concentration as described previously^[118]. The four groups were fed *ad libitum* for 12 weeks.

Immunoblotting

Rats were lightly anesthetized (Dolethal, 200 mg/Kg Vetoquinol S.A., Madrid, Spain) and then killed by decapitation. Brains were rapidly removed and frozen in cold isopentane (Sigma-Aldrich, St Louis, MO, USA). Hippocampi were dissected at -15 °C (using a cryostat) to preserve the protein phosphorylation. Tissue was lysed in a RIPA buffer containing protease and phosphatase inhibitors (SERVA Electrophoresis, Heidelberg, Germany). Mechanical tissue lysis was achieved using a sonicator (Hielscher Ultrasound Technology, Teltow, Germany). Thirty micrograms of the total protein were subjected to SDS-PAGE, transferred to Immobilon-P membranes (MERCK Millipore, Darmstadt Germany), which were blocked for nonspecific binding and incubated with primary antibodies: anti-phospho IKK α β (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500); anti-phospho TAU (Abcam, Cambridge, UK; 1:1000); anti-IRS1 (EMD Millipore, Temecula, CA, USA; 1:500); anti-IRS2 (Cell Signaling, Danvers, MA, USA 1:1000), and anti- β -Actin (Sigma-Aldrich, St Louis, MO, USA; 1:2000) overnight at 4°C. After several washes with a washing buffer containing 0.3% Triton X-100, the membranes were incubated for 1 h at RT with peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, Jackson ImmunoResearch, Suffolk, UK). Staining was developed using ECL (BioRad, Hercules CA, USA), and digital images were captured with a charge-coupled device imager (IMAGEQUANT LAS 4000, GE Healthcare Little Chalfont, UK). Immunoreactive bands were quantified with Image J blots toolkit software (National Institutes of Health, Baltimore, MD, USA), which was normalized to the β -Actin signal in each sample. Data were expressed as a percentage of the control (standard diet) normalized signal as the mean plus and minus the standard error of mean (SEM).

Immunohistochemistry

Rats were anesthetized with pentobarbital (120 mg/kg Eutanax, Fatro, Barcelona, Spain) and transcardially perfused with saline (0.9%), followed by a 4% PFA fixative in 0.1 M phosphate buffer, pH 7.4. After perfusion, the brains were removed and post-fixed overnight at 4°C in PFA, followed by 48 h in a 30% sucrose solution for cryoprotection. Sliding Microtome Leica SM2010R (Leica Microsystems, Heidelberg, Germany) was used to obtain 40- μ m thick coronal frozen sections. The brains were cut in rostrocaudal direction; six series of slices were collected from each brain and stored at -20 °C. Sections were rinsed twice in 0.05 M TBS, pH 8.0, containing 0.2% Triton X-100 at RT. Slices were incubated in blocking solution (containing 2% donkey serum and 4% serum albumin bovine for 1 h); anti-Doublecortin (DCX) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000) was used. After several washes, the slides were incubated with the biotinylated secondary antibody (Jackson 1: 200) in TBS with 0.2% Triton X-100. Then sections were rinsed 3x for 10 min and transferred to a 1:50 avidin–biotin–horseradish peroxidase complex solution for 90 min (VECTASTAIN® Elite® ABC-HRP Kit; Peroxidase, Standard, USA). Then, sections were rinsed 2x for 10 min in TBS and 2x for 10 min in 0.05 M 7.6 Ph Tris Buffer (TB). Color reaction was achieved by incubation with DAB (Sigma-Aldrich, St Louis, MO, USA) and 2

μl of H_2O_2 in TB for 15-20 min. The reaction was stopped by several rinses in phosphate buffered saline solution. Finally, the slices were mounted on gelatinized slides and air dried overnight. Then, sections were rehydrated, alcohol-dehydrated, xylene-cleared and coverslipped with a DPX mounting medium.

Image Analysis

Images were taken by a Nikon optical microscope (Nikon, Tokyo, Japan) attached to a Leica camera (Leica Microsystems), which was connected to the Leica software (Leica Microsystems), to acquire the images. To quantify the DCX-positive neurons, six images were taken for each anatomical subdivision of the HPC in the four treatments (HFD, HFD-ABA, SD, and SD-ABA). The DCX-positive neurons were counted with Image J (National Institute of Health, Baltimore, MD, USA) at levels rostral (Bregma -3.72 mm) and caudal (Bregma -6.00 mm) levels (Paxinos and Watson^[284]). Data were expressed as the mean and the SEM of the DCX-positive neurons ($n = 6$). The researcher performing the quantification of DCX-positive neurons was blind to the experimental condition.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the rat HPC ($n= 5-11$) and homogenized in 500 μL of lysis buffer according to the Norgen Fatty Tissue RNA Purification Kit (Norgen Biotek Corp, product no. 36200, ON, Canada). Genomic DNA was removed using a spin-column process during the RNA extraction. In addition, DNase I treatment (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was performed to ensure the complete removal of genomic DNA. RNA samples were eluted in 50 μL of nuclease-free water and reverse transcribed to cDNA using a PrimeScriptTM RT reagent kit (Takara, Shiga, Japan) following the manufacturer's instructions. Primers were designed using the Primer3 software tool (<http://primer3.ut.ee/>) (Table 4.1). RT-qPCR reactions were carried out using SYBR PREMIX Ex Taq (Tli RNase H Plus) (TAKARA Bio Inc., Shiga, Japan) in an Applied Biosystems StepOne PlusTM Real-Time PCR System (Foster City, CA, USA). At the end of each PCR reaction, a melting curve stage was performed to confirm that only one PCR product was amplified in these reactions. The relative gene expression to SD was calculated by using the $2^{-\Delta\Delta\text{Ct}}$ method for each reaction and by using the housekeeping gene GAPDH as internal control.

Table 4.1. Primers sequences obtained from Primer3 software tool.

Genes	Accession number	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	NM_017008.4	TGCCCCATGTTTGTGATG	TGGTGGTGCAGGATGCATT
INSR	M29014.1	TCCTAAAGATCCGTCGCTCC	AAGAGCTTGCCCTGAGTGAT
IGF-1R	NM_052807.2	TGTCCTCTCGGCATCAAACCT	TATCACCACCGCACACTTCT
IRS-1	NM_012969.1	ATTGGAGGTGGGTCTTGCAG	TGGGGATCTTCTGGGCCATA
IRS-2	NM_001168633.1	TCACCACAGGACACAGATGC	GCATGAAGTGTGGCAAACGT
APP	NM_019288.2	CCCCAAGATCCGGTTAAACT	TACTTGTCGACTGCGTCAGG
BDNF	NM_001270638.1	GAGACAAGAACACAGGAGGAAA	CCCAAGAGGTAAAGTGTAGAAGG
TNF α	NM_012675.3	GACCCTCACACTCAGATCATCTTCT	TGCTACGAGGTGGGCTACG

Statistics

Data were expressed as mean \pm SEM and subjected to a Kolmogorov-Smirnov normality test, and only when the test was positive for normality ($\alpha = 0.05$) were the data analyzed using a parametric one-way ANOVA followed by a post-hoc Newman-Keuls test. To evaluate the associations between variables, we used a two-tailed Pearson correlation test. The correlation coefficient r values (± 0.1 to ± 0.3) reveal a weak association, are a (± 0.3 to ± 0.5) medium, and

are higher than ± 0.5 strong correlation^[285–287]. Positive or negative r values mean positive or negative correlations, respectively^[288].

4.3. Results

4.3.1. A HFD alters hippocampal IRS1 and IRS2 mRNA expression. ABA treatment can prevent these changes

We found that TNF α levels were increased significantly by HFD treatment, and ABA administration, along with a HFD, prevented this significant increase ($F_{3,39} = 9.363$). Similarly, we observed that the BDNF mRNA level in the HPC was significantly reduced in HFD-treated animals compared to SD and that the treatment with ABA in the drinking water prevented this reduction ($F_{3,16} = 5.898$). Interestingly, the APP mRNA expression levels also showed a significant increase with HFD exposure ($F_{3,19} = 4.524$).

Next, we wanted to evaluate the effects of a HFD on the proteins' expression of the insulin-signalling pathway. Even though it is widely accepted that a HFD and neuroinflammation induces insulin resistance, whether a HFD affects mRNA levels of IR, IRS1, and IRS2 is largely unknown. In our model, we observed a significant reduction of IRS2 mRNA levels in the HPC of rats fed a HFD with respect to SD (Table 4.2). Most interestingly, ABA treatment prevented this significant reduction in the hippocampal IRS2 levels in rats fed a HFD ($F_{3,37} = 5.911$). Moreover, there was a significant increase in the mRNA levels of IRS1 in those animals fed a HFD, which can also be rescued by ABA administration ($F_{3,22} = 4.301$) (Table 4.2). Hence, these results suggest that the modulation of IRS1 and IRS2 gene expression may be different, depending on the environmental conditions, which suggests that these proteins may be involved in different cellular needs, in insulin signalling, and in neuroinflammation. IR did not change with diet or ABA treatment; however, the IGF-1R did show a change in those control samples that were treated with ABA ($F_{3,16} = 3.025$) (Table 4.2).

Table 4.2. Hippocampal mRNA expression of neuroinflammation markers and Insulin/IGF-1 pathway genes.

Genes	HFD	HFD-ABA	SD	SD-ABA
TNF α (n=7-15)	1.67 \pm 0.16	1.19 \pm 0.14 *	1.01 \pm 0.04 ***	0.99 \pm 0.04 **
BDNF (n=5)	0.59 \pm 0.04	0.85 \pm 0.10	1.00 \pm 0.02 **	0.87 \pm 0.09
APP (n=5-6)	1.8 \pm 0.29	1.41 \pm 0.22	1.00 \pm 0.01**	0.95 \pm 0.13 *
IRS1 (n=7)	1.28 \pm 0.08	1.06 \pm 0.04	1.01 \pm 0.02*	1.06 \pm 0.08
IR (n=5-7)	0.97 \pm 0.07	0.95 \pm 0.14	1.01 \pm 0.01	0.99 \pm 0.04
IGF-1R (n=5)	1.24 \pm 0.14	1.11 \pm 0.10	1.00 \pm 0.01	0.80 \pm 0.13*
IRS2 (n=7-14)	0.59 \pm 0.06	1.02 \pm 0.09 *	1.00 \pm 0.06 **	0.83 \pm 0.15

4.3.2. ABA can effectively restore HFD-induced reduction in hippocampal IRS2 protein

To evaluate the effect of a HFD on protein expression in the HPC, we measured the levels of both IRS1 and IRS2 and the inflammatory markers phosphorylated inhibitor of nuclear factor kappa-B kinase subunit (pIKK)^[289] and TAU (pTAU)^[290] using a western blot (Fig. 4.1A). We observed that IRS2 protein levels were significantly lower in the HFD group (0.61 \pm 0.05; n = 11) compared to SD group (0.99 \pm 0.02; n = 9), HFD supplemented with ABA group (1.00 \pm 0.16; n = 12), and the SD-ABA group (1.29 \pm 0.17; n = 7), ANOVA ($F_{3,35} = 4.897$; $p = 0.006$) (Fig. 4.1B). In contrast, hippocampal IRS1 protein levels from matched samples were not different from each other [HFD (0.97 \pm 0.12; n = 5), HFD-ABA (1.05 \pm 0.12; n = 5), SD (0.96 \pm 0.02; n = 5), SD-ABA (1.31 \pm 0.25; n = 5)] (Fig. 4.1C). On the other hand, pIKK did not show any significant differences

between groups [HFD (1.38 ± 0.32 ; $n = 6$), HFD-ABA (1.28 ± 0.20 ; $n = 6$), SD (0.98 ± 0.01 ; $n = 7$), SD-ABA (1.15 ± 0.07 ; $n = 7$)] (Fig. 4.1D). We found that ABA increased pTAU expression in SD-ABA animals (1.37 ± 0.19 ; $n = 7$) with respect to the SD group (0.99 ± 0.02 ; $n = 9$), that the HFD-treated animals had lower levels, although not significantly lower (0.68 ± 0.12 ; $n = 11$), and ABA added to HFD group (0.88 ± 0.07 ; $n = 11$) did not significantly alter the pTAU levels, ANOVA ($F_{3,34} = 6.012$; $p = 0.002$) (Fig. 4.1E).

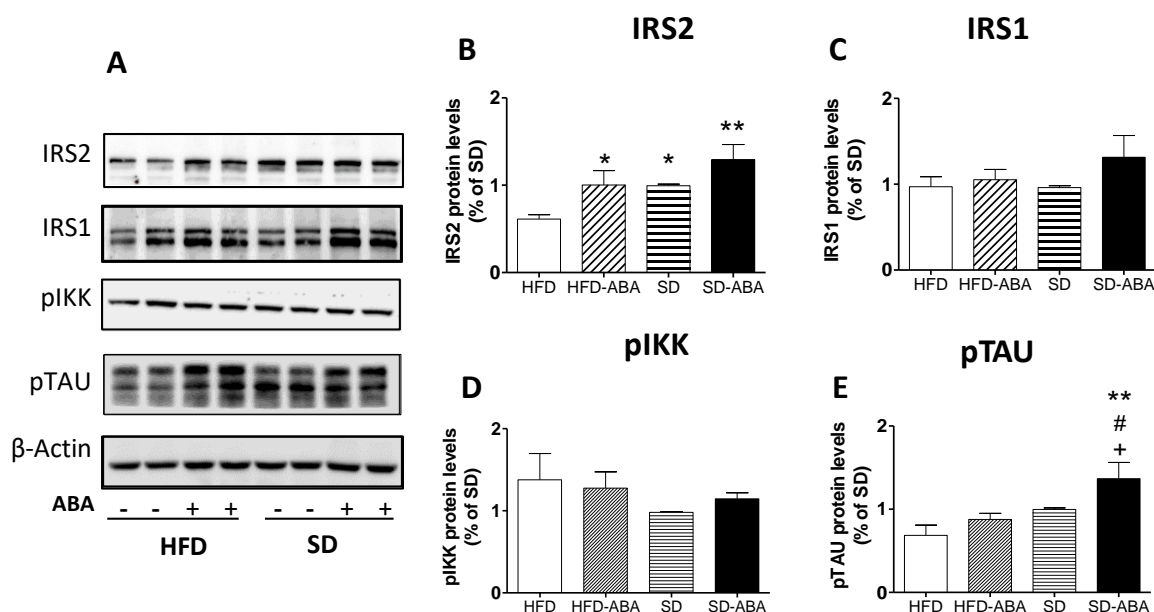


Fig. 4.1. A western blot analysis was carried out to evaluate the expression of the proteins in the hippocampus. Representative images of IRS2, IRS1, pIKK, pTAU, and loading control β -Actin (A). Quantifications of IRS2 protein levels (B), IRS1 (C), pIKK (D), and pTAU (E). HFD (white columns); HFD-ABA (diagonal lines); SD (horizontal lines), and SD-ABA (black columns). Data are represented as the mean \pm SEM ($n = 6$). The ANOVA was followed by a post-hoc Newman-Keuls test ($n = 5-11$). *, $p < 0.05$; **, $p < 0.01$ vs. HFD; #, $p < 0.05$ vs. HFD-ABA; +, $p < 0.05$ vs. SD.

4.3.3. ABA rescues the HFD-induced reduction in hippocampal neurogenesis

To evaluate hippocampal neurogenesis, we quantified DCX, which was expressed in differentiating and migrating neurons during embryonic and postnatal development^[291] using immunohistochemistry in the rostral (Bregma -3.72 mm) and caudal HPC (Bregma -6.00 mm) (Fig. 4.2A), following the rat brain atlas^[284]. Data were expressed as the mean of DCX-positive neurons \pm SEM. In the rostral HPC, we found that the number of DCX-positive neurons decreased significantly in HFD-fed rats (131.9 ± 41.4 ; $n = 6$) compared to SD-fed controls (275 ± 34.15 ; $n = 6$). The ABA treatment rescued the DCX expression in HFD-ABA animals (339.1 ± 43.99 ; $n = 6$) and did not alter the DCX in SD animals (257.2 ± 34.15 ; $n = 6$). ANOVA ($F_{3,20} = 6.150$; $p = 0.0039$) (Fig. 4.2B). There were representative images of DCX-positive neurons in HFD (Fig. 4.2C), HFD-ABA (Fig. 4.2D), SD (Fig. 4.2E), and SD-ABA (Fig. 4.2F). An identical pattern was followed in the caudal HPC (data not shown).

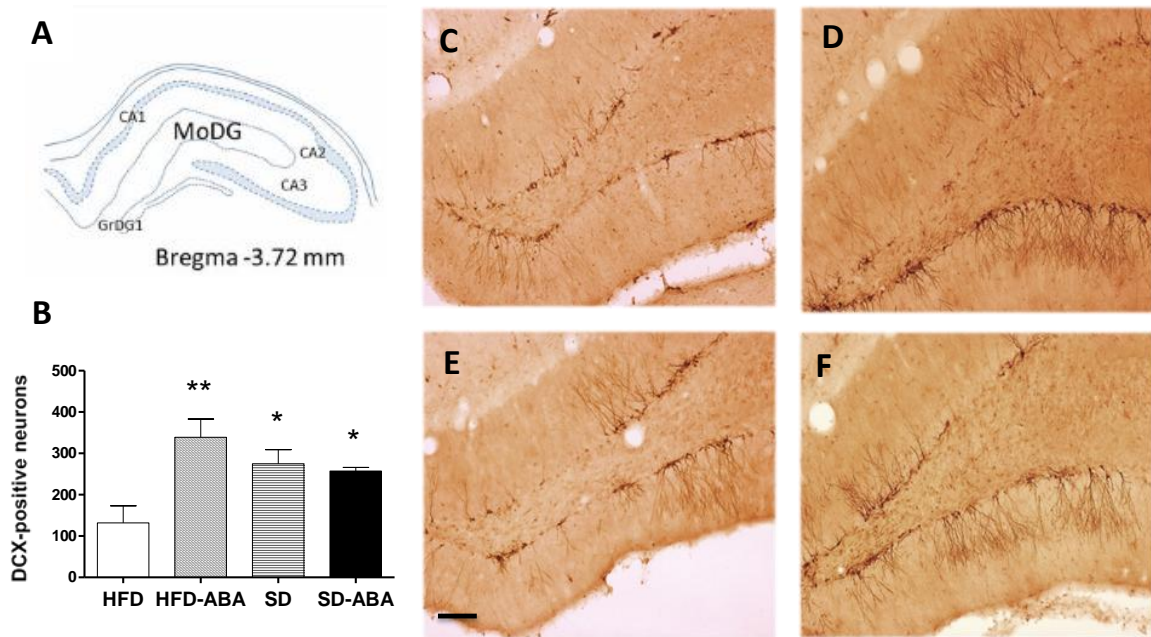


Fig. 4.2. ABA rescues the HFD-induced reduction in the number of DCX-positive neurons in the hippocampus. A Patxinos drawing of a rostral hippocampus (Bregma - 3.72 mm) (A). The quantification of DCX-positive neurons (B). Data are represented as the mean \pm SEM ($n = 6$) and analyzed using a one-way ANOVA followed by post-hoc Newman-Keuls test. *, $p < 0.05$; **, $p < 0.01$ with respect to HFD. The representative images are of DCX immunolabeling in HFD (C), HFD-ABA (D), SD (E), and SD-ABA (F). Calibration bar, 100 μm .

4.3.4. *IRS1* and *IR21* mRNA levels correlate differently with *TNF α* , *BDNF* and *APP*

We have observed that diet and ABA treatment can differently affect the expression of *IRS1* and *IRS2*. To further understand the relationships of *IRS* expression and other biomarkers of inflammation, we aimed to ascertain if there was a relationship between the levels of *IRS1* and *IRS2* gene expression with *TNF α* , *APP*, and *BDNF*. We observed that *IRS1* correlates positively with *TNF α* (Pearson $r = 0.574$; $p = 0.02$) (Fig. 4.3A), whereas *IRS2* correlates negatively with *TNF α* (Pearson $r = -0.498$; $p = 0.05$) (Fig. 4.3D). Interestingly, *IRS1* showed a positive correlation with *APP* (Pearson $r = 0.672$; $p = 0.003$) (Fig. 4.3B). In contrast, *IRS2* did not correlate with *APP* either way (Fig. 4.3E). None of the substrates showed any correlation with *BDNF* levels (Fig. 4.3C and F, respectively).

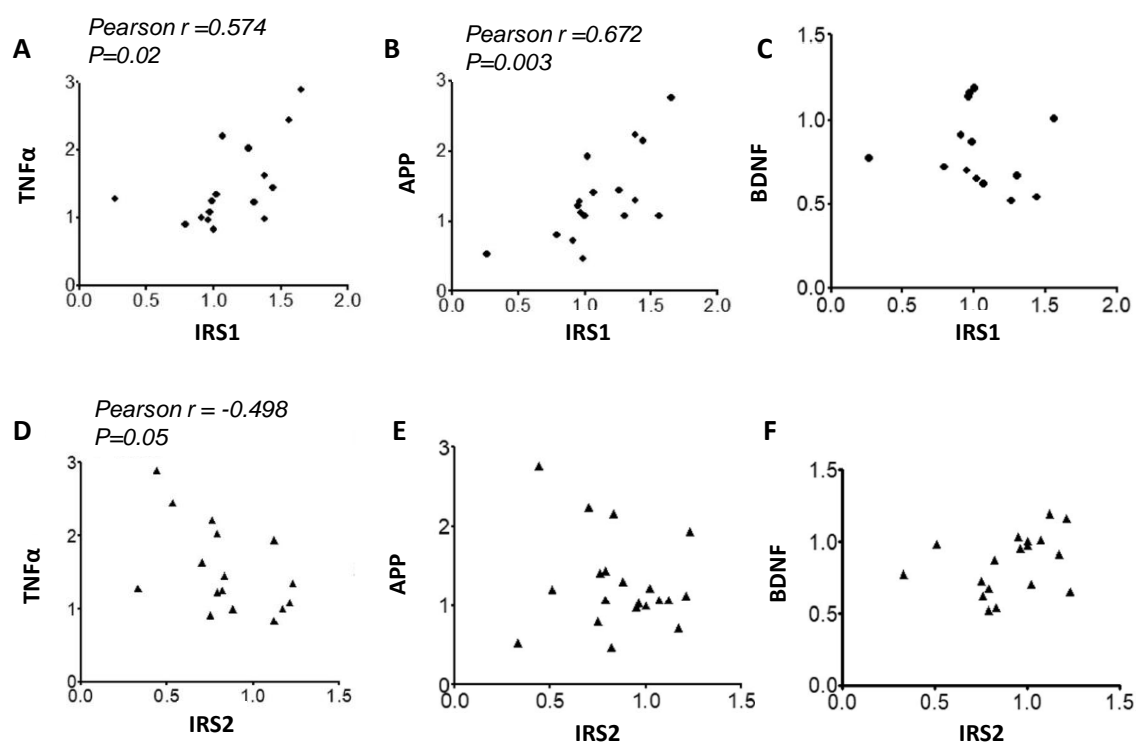


Fig. 4.3. The correlation of IRS1 and IRS2 with TNF α , APP, and BDNF. The IRS1 expression correlates positively with the TNF α (A) and APP (B) expression. IRS1 does not correlate with the BDNF expression (C). The IRS2 gene expression correlates negatively with TNF α (D) and does not correlate with the APP (E) or BDNF (F) expression.

4.3.5. The IRS1 expression correlates with weight gain but IRS2 does not

Due to the different patterns of expression showed by both isoforms of IRS, we aimed to elucidate if there was a relationship between the percentage of weight gain and the IRS expression. Interestingly, IRS1 showed a positive correlation with the percentage of weight gain (Pearson $r = 0.498$; $p = 0.019$) (Fig. 4.4A). However, the IRS2 expression did not show any significant correlation when plotted versus the percentage of weight gain (Fig. 4.4B).

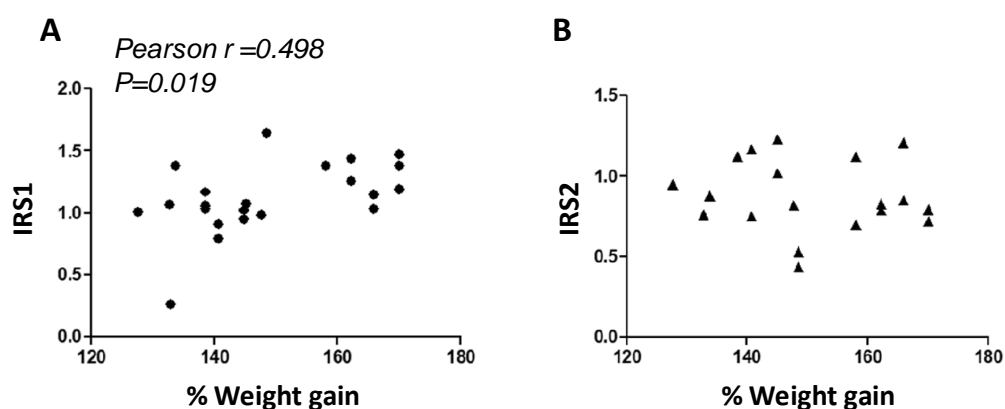


Fig. 4.4. The correlation of IRS1 and IRS2 versus the percentage of weight gain. The IRS1 expression correlates positively with the percentage of weight gain (A). There is no significant correlation between IRS2 and the percentage of weight gain (B).

4.4. Discussion

Excessive caloric intake can induce neuroinflammation and insulin resistance. Both processes are considered major risk factors that lead to cognitive alterations ^[219,220,292].

In this study, we have made use of a well-established model of neuroinflammation -feeding rats a HFD for 3 months- to study its effects on the insulin-signalling pathway. In addition, we have evaluated the effects of the phytohormone ABA in hippocampal insulin signalling. ABA targets PPAR γ in a manner similar to the TZDs class of anti-diabetic drugs ^[222,293] and curcumin, a well-known potent anti-inflammatory molecule ^[294] and also PPAR γ agonist ^[295]. In fact, given their capability to reduce inflammation, this family of PPAR γ agonist molecules has been proposed as a new class of molecules with which to treat central nervous system disorders ^[296].

We have analyzed the mRNA of IR and IGF-1R and their substrates, IRS1 and IRS2. We found no changes in hippocampal IR or IGF-1R in the HPC of HFD-fed animals compared to the controls. However, our study shows for the first time that a HFD can reduce IRS2 expression in hippocampal tissue, both at the messenger RNA and protein levels, therefore providing an additional plausible mechanism of hippocampal insulin resistance in obesity and metabolic syndrome. These results are also in line with previous reports showing that IRS2 is reduced in dorsal root ganglia from diabetic (insulin resistance) rodent models ^[297]. Moreover, in the gastrocnemius muscle, IRS2 expression was found to be significantly reduced in diabetic animals compared to the controls ^[298]. On the contrary, we observed that HFD increased IRS1 levels. These results may be paradoxical; however, they are in line with earlier reports indicating that IRS1 mRNA is increased in the HPC of APP/PS1 (presenilin 1) transgenic mice compared to aged-matched controls ^[299].

Interestingly, we have observed that treatment with ABA restored IRS1 and IRS2 expression, which could account for beneficial effects improving the cognitive performance of HFD-fed animals ^[118]. These results are supported by studies showing that PPAR γ agonists can restore pathological IRS levels, that curcumin reduced the IRS1 levels in double APP/PS1 transgenic mice ^[299], and that pioglitazone increased the pancreatic IRS2 expression in a model of diabetic transgenic mice ^[144].

To our knowledge, our study reports for the first time that HFD alters hippocampal IRS1 and IRS2 expression and that supplementation with ABA is capable of restoring these alterations.

Protein expression was studied using immunodetection and confirmed the alterations observed in the IRS2 mRNA levels, but not IRS1 mRNA. This discrepancy in messenger and protein expression could be since the IRS1 protein levels may be regulated by degradation, and, therefore, the increase in mRNA is not being detected in the protein. Paradoxically, pIKK and pTAU were not found to be increased in a HFD as expected given the fact that inflammation augments the phosphorylation of IKK ^[300,301] and that insulin resistance results in tau hyperphosphorylation ^[302]. Nevertheless, supporting our results, pIKK levels are not altered in HFD-treated rats ^[303], indicating that pIKK may not be a universal and reliable marker for neuroinflammation. However, further studies should be carried out to elucidate the role of pIKK in hippocampal HFD-induced inflammation. Similarly, future research will help to understand the conditions required for accumulation of pTAU derived from an insulin-resistance situation. It is plausible that time is an important factor for observing such accumulation.

Furthermore, we have observed that ABA treatment is capable of restoring a HFD-induced reduction in neurogenesis, as evaluated by DCX-positive neurons in the subgranular layer of the dentate gyrus. It is well accepted that a HFD reduces neurogenesis and that exercise and learning can improve it ^[304]. Consistent with our data, many insulin sensitizer molecules used against type 2 diabetes have been proven effective in restoring altered neurogenesis ^[305]. In addition, BDNF signalling is closely related to neurogenesis modulation, synaptic plasticity, and the formation and recall of hippocampal-dependent memories ^[306]. BDNF reduction has also been proposed as one of the earliest events in HFD-induced cognitive impairment ^[307,308]. In this study, in addition to

showing impaired neurogenesis, we observed that the BDNF mRNA levels were reduced in the HPC with HFD and that ABA treatment could efficiently prevent this reduction. This is in line with previous studies reporting that neurogenesis and BDNF levels can be rescued using insulin and BDNF-sensitizing nutritional compounds^[309] and anti-inflammatory supplementation^[310]. BDNF may be able to induce IRS1 and IRS2 tyrosine phosphorylation^[311] but, to date, it has not been reported to regulate IRS1 or IRS2 expression. In fact, we found no correlation between IRS1 and IRS2 with BDNF levels.

On the other hand, TNF α , a well-accepted marker of neuroinflammation, insulin resistance, and cognitive impairment^[312], increases in the brain of HFD-treated animals^[313]. As expected, we found that TNF α levels were increased in the HPC of HFD-treated rats and that ABA treatment significantly could prevent it. This result confirms that ABA is an effective anti-neuroinflammatory molecule. Furthermore, since TNF α levels are also closely linked to impaired neurogenesis and reduction in BDNF^[314], our study confirms the close relationship of these processes with IRS expression.

APP transgenic mice have been extensively used as a model for AD in an attempt to over-express a mutated protein that promotes oligomer aggregation^[315]. We have found that endogenous APP levels are increased in the HPC of HFD-fed rats. It is plausible that this increment can favour A β accumulation^[316] and mediate some of the deleterious effects of a HFD in cognition. We found that ABA treatment could effectively counteract these effects, preventing the overexpression of APP. Other studies have also shown that supplementary nutrients can effectively downregulate APP endogenous expression^[317]. Therefore, the anti-inflammatory and insulin-sensitizing actions also regulate APP expression, preventing further complications of A β deposition.

Finally, to understand the relationship between IRSs, the biomarkers used in this study and obesity, we analyzed the normalized levels of IRS1 and IRS2 with TNF α , APP, BDNF rat hippocampal tissue, and the percentage of weight gain. Interestingly, we observed the levels of TNF α correlate inversely with the expression of IRS2 and positively with IRS1. This fact strongly suggests that IRS1 and IRS2 can be differentially affected by neuroinflammatory processes and therefore modulates distinct aspects of insulin resistance. Further confirming this hypothesis, we found that APP correlates positively with IRS1 but does not correlate at all with IRS2; thus, it is plausible that the signalling pathway that affects APP can target only IRS1 but not IRS2. Moreover, the percentage of weight gain correlates positively with IRS1 but not with IRS2 expression.

Considering all these data, we conclude that both IRS1 and IRS2 expression regulation, although modulating insulin and IGF-1 signalling, are totally independent of each other, suggesting that their implication in neuroinflammation and brain insulin resistance may also be different. Further studies should be carried out to establish the mechanism underlying these differences, such as location, protein interactions, and promoter sensitivity. These findings will be crucial for opening different methods of brain insulin resistance treatment.

Moreover, we confirm that ABA can effectively restore neuroinflammation and neurogenesis, supporting the beneficial effects on memory that we have reported previously^[118]. In addition, we show that ABA can also influence IRS1 and IRS2 expression levels, providing a mechanistic link to how ABA can restore insulin signalling.

5

Chapter 3

AAV DELIVERY OF SHRNA AGAINST IRS1 IN
GABAERGIC NEURONS IN RAT HIPPOCAMPUS
IMPAIRS SPATIAL MEMORY IN FEMALES AND
MALE RATS

5.1. Introduction

Insulin and IGF-1 actions are mediated by the insulin and IGF-1 receptors that can activate similar signalling pathways with pleiotropic actions in mammals. In the brain, both peptides have been shown to modulate cognitive function^[318,319]. The signalling cascades elicited by insulin and IGF-1 are almost identical and both mediated by the scaffolding proteins, the IRS^[71,272]. There are four IRSs known isoforms, being the IRS1 and IRS2 the most abundant and studied, up to date^[272]. In spite the fact that both, IRS1 and 2 have been shown to mediate insulin and IGF-1 effects, some reports reveal that these isoforms may modulate different signalling cascades in the liver and skeletal muscle tissues^[70,320]. Also, studies using knockout mice suggest that IRS1 and IRS2 modulate different physiological processes^[320–324]. However, these differences have not been fully established within the central nervous system, and some paradoxical results indicate that whereas brain insulin resistance is mediated by dysfunctional IRS1^[75] in other systems the reduction of the IGF-1/IRS2 pathway is suggestive of a neuroprotective role^[325,326]. In optimal conditions, both IRSs are considered equally activated by the insulin and the IGF-1 receptors tyrosine kinase activity. Once these scaffolding proteins are phosphorylated in Tyr residues, they activate downstream signalling cascades, e.g. the PI3K/Akt pathway, Ras/ERK; and soluble tyrosine kinases from the Fyn family^[327–333]. Several physio-pathological conditions induce resistance to insulin and IGF-1. Among others, neuroinflammation feed-forward inhibition induces IRS1 phosphorylation in serine residues by cytokines like IKK β and JNK. In hyperinsulinemia, feedback inhibition via mTOR also phosphorylates IRS1 in Ser residues^[47,48,334]. Interestingly, both chronic insulin and IGF-1 resistance in the brain are associated with the occurrence of AD^[335,336]. Furthermore, reduced responses to IR-IRS1 and IGF-1R-IRS2 linked to PI3K^[76] have been found in postmortem analysis in AD patients, compared to controls.

The specific and differential role of IRS1 or IRS2 isoforms in distinct brain networks, isolated from other comorbidities, such as chronic inflammation or AD linked genetic mutations, has not been fully established. Whether insulin resistance is a primary cause of cognitive dysfunction or it is a consequence of inflammatory conditions remains elusive. Thus, we aimed to understand the specific role of IRS1 in the adult healthy brain, in the absence of any other peripheral or central comorbidities. To that end, we made use of adeno-associated virus (AAV) expressing short hairpin ribonucleic acid (shRNA) targeting IRS1 (shIRS1) into the dorsal HPC of female and male rats. A rat model has been extensively used to study brain function with well-accepted methods of behavioural evaluation. We evaluated the IRS1 silencing effect in specific hippocampal circuitries involved in spatial memory and the synaptic plasticity of infected neurons. We report for the first time that, downregulation of IRS1 in dentate gyrus hilar PV and SOM neurons impairs spatial memory but not induces anxiety or depressive symptoms. In addition, our results suggest that female rats may be more susceptible to brain insulin resistance than males, which may contribute to explain sex differences in the prevalence of neurodegenerative diseases.

5.2. Materials and Methods

Design and generation of shRNA constructs

Several sequences specifically targeting IRS1 were designed using the provided software on the Invitrogen webpage (<https://rnaidesigner.thermofisher.com/rnaiexpress/>); parameters were modified to fit destination plasmid for shRNA cloning (pSM155). Best shIRS1 candidates were synthesized (ThermoFisher Scientific®). Two shRNA constructs were cloned following the protocol described^[337]. The sequences of the oligonucleotides used in this study are shown in Supplementary Figure S5.1 (annexes section).

Construction and purification of recombinant AAV-CMV-shIRS1A-EGFP

To generate the expression cassette, a PCR (*Supreme NZY-Proof DNA polymerase, NZYtech, Lisbon, Portugal*) targeting the shIRS1 and the enhanced green fluorescent protein (EGFP) was performed. Primers were designed with the *Clal* and *HindIII* restriction sites. The PCR product was cloned into pJET 1.2 according to the manufacturer's instructions (*CloneJET PCR Cloning Kit; ThermoFisher Scientific®, MA, USA*). Insert was cloned by *Clal/HindIII* enzyme restriction from the pJET 1.2 constructs and subcloned into the destination vector pAAV-CMV-GFP (*Cell Biolabs INC, CA, USA*) by ligation (*T4 DNA Ligase; Takara Bio INC, Kyoto, Japan*) overnight. Ligation was transformed into *Endura™* competent cells (*Lucigen, WI, USA*), and positive colonies evaluated by *MscI* and/or *EcoRV* enzyme digestion. Finally, sequencing confirmed the absence of point mutations (Servicio Central Soporte Investigacion Experimental from the University of Valencia). pAAV-CMV-shIRS1A-EGFP was co-transfected with pHelper and pAAV2-Dj/8 (*Cell Biolabs, INC.*) into packaging Human embryonic kidney cells 293 (HEK293) cells to produce recombinant AAV2/DJ8-CMV-shIRS1A-EGFP. The resulting viral particle was purified by the iodixanol method as described in *Addgene protocols for AAV production and purification*^[338]. Cell lines were purchased from *Cell Biolabs, INC*, and were free from mycoplasma.

Animals and surgical procedures

Female and male Wistar rats (Janvier Labs, Saint-Berthevin, France) between 250 and 550 g were kept at the animal facility of the University Jaume I. The procedures followed the directive 86/609/EEC of the European Community on the protection of animals used for scientific purposes. The experiments were approved by the Ethics Committee of the University Jaume I (scientific procedure 2019/VSC/PEA/0143). The animals were maintained on a 12 h light cycle and provided with food and water *ad libitum*. Animals were caged in pairs to reduce single caging -induced stress. Surgeries were done over one week, approx. 4 surgeries a day. Female and male cohorts were done in different phases to avoid confounding results.

For surgical procedures rats were first deeply anesthetized with ketamine (Ketamidor 50 mg/kg i.p.; Merial Laboratories SA, Barcelona, Spain) and xylazine (Xilagesic 10 mg/kg i.p.; Lab Calier, Barcelona, Spain). Stereotaxic coordinates used to infuse the viral particle into the dorsal HPC bilaterally were AP -4.36 mm, ML \pm 2.5 mm, DV -2.5 mm from bregma^[284]. Bilateral injections were performed; 2 μ L of either AAV/DJ8-CMV-EGFP (4.94E+11 GC/mL) or AAV/DJ8-CMV-shIRS1-EGFP (1.02E+11 GC/mL) per hemisphere. Animals were randomly divided into AAV control AAV shIRS1 groups and code labelled by an independent researcher. We used 7-8 rats per group and sex. This group size has been chosen based on our previous experience and the literature. Animal welfare was monitored although the procedure according to Ethical committee rules. Table and graph showing the subject's weight before the surgery and one month later, before behaviour tests are shown in Supplementary Table S5.1 (annexes section).

Behavioural procedures

In all the procedures rats were habituated to the testing room two days before performing the behaviour paradigm. Animals were recorded using a video tracking system (Smart 2.5.19, Panlab,

Barcelona, Spain). Behavioural tests were conducted during the day, with a dim light. The researcher was blind to the group's condition. All animals performed these tests.

Open Field; Rats were placed in the open field facing one of the walls and allowed to freely explore the arena for 10 min. The latency (time) to cross for the first time the centre quadrants with all four legs was quantified.

Novel Object Recognition (NOR); NOR experiment was conducted as described previously^[118]. Briefly, rats were habituated to the experimental box before the test day. In the test day, the subjects were left to explore two identical objects, for 3 minutes (familiarization phase). After 1h interval, the rats were back to the arena, and allowed to explore one of the previous objects (familiar) and a novel object for 3 min (test phase). "Exploration" is defined as time spent sniffing the object closer than 4 cm, or touching it with the head oriented towards the object. Climbing over the object or running around it was not considered exploration. Data were expressed as the Discrimination Index (DI), defined as the time spent in the novel object, minus time spent in familiar one, divided by total time exploring. Thus, DI =0 means the animal spends equal time exploring both objects. DI=1 or DI= -1 would mean that the subject spends total time exploring the novel or the familiar, respectively.

T-maze; The T-maze test was performed as described previously^[118]. Briefly, the animal is placed in the starting position and left to explore for 5 min, with access to two of the three arms (familiarization phase). The rats were then returned to the home cage for a 2-h inter-trial interval and then placed back in the starting position, but now with access to all three arms for 5 min (test phase). The arm that was closed during the familiarization phase is considered the "novel" arm, the arm visited during the familiarization phase, is considered the "familiar" arm. Data expressed as the DI, as above.

Forced Swim Test; The forced swim test (FST) apparatus consisted of a Plexiglass cylinder with translucent walls (30 cm in diameter and 50 cm high). The cylinder was filled with tap water at 23 ± 1 °C and the water depth was adjusted so the rat cannot touch the bottom of the container. In the first session (pre-test phase), the rats were placed in the water-filled cylinder for 15 min. Twenty-four hours later (test phase), each rat was returned to the FST apparatus for 5 min^[339]. Once each session was finished, the animals were placed in a drying cage with a heating pad under it and constant temperature that did not exceed 37 °C. Both days, the cylinder was placed at the same position in the room and the water was changed after every test to avoid any influence on the next rat. Three parameters were measured: "Immobile" was considered when the rat was floating without any movement, "Climbing" when the limbs were moved quickly breaking the surface of the water, "Swimming" when the rat moved the limbs in a paddling way.

Immunofluorescence

Immunofluorescence was performed as described previously^[114]. Briefly, randomly chosen rats were anesthetized and transcardially perfused with saline (0.9% NaCl) followed by fixative (4% paraformaldehyde in 0.1 M PB, pH 7.4). The other half of the brains was frozen for biochemical analysis. However, given the specificity of the AAV infection, considering that the surrounding neurons do express mRNA IRS1 we did not perform biochemical analysis.

After perfusion, the brains were removed, postfixed overnight and cryoprotected in 30 % sucrose in 0.01 M PBS pH 7.4 for 3 days. The brains were cut in rostrocaudal direction (40 µm) using a sliding microtome Leica SM2010R (Leica Microsystems, Heidelberg, Germany). The following primary antibodies were used: goat anti-ChAT (MERCK Millipore, Darmstadt Germany; 1:700); mouse anti-calbindin (CB) (Swant, Marly, Switzerland; 1:3000); rabbit anti-calretinin (CR) (Swant; 1:2000); rabbit anti-parvalbumin (PV) (Swant; 1:5000); mouse anti-somatostatin (SOM) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50); mouse anti-synaptophysin (Sigma-Aldrich, St Louis, MO, USA; 1:100); mouse anti-Glial Fibrillary Acidic Protein (GFAP) (Sigma-Aldrich, St Louis, MO, USA; 1:100); rabbit anti-IRS1 (Novus Biologicals, Abingdon, UK; 1:100). After overnight incubation, AAV-infected sections were rinsed and incubated for 2 h at RT with

donkey anti-mouse Cy3, donkey anti-goat Cy3, and goat anti-rabbit Cy3 secondary antibodies (Jackson ImmunoResearch, Suffolk, UK). Finally, sections were mounted on slides and coverslipped using Fluoromount-G mounting medium (Invitrogen, California, USA). For descriptive immunofluorescence, three animals per condition were done, and one example is shown in results. For IRS1+EGFP dual-labeling quantifications female rats 3 controls and 4 shIRS1 and male rats 7 controls and 7 shIRS1 were used and 2 dentate gyri per animal. For CR+AAV, PV+AAV, and SOM+AAV dual-labelling quantifications female rats 4 shIRS1 and male rats 7 shIRS1 were used and 1 dentate gyrus per animal. For synaptophysin quantifications female rats 3 controls and 4 shIRS1 were used and 10 different axonal fibers per animal.

Imaging and analysis

Fluorescence images were taken with a confocal scan unit with a module TCS SP8 equipped with argon and helium-neon laser beams attached to a Leica DMI8 inverted microscope (Leica Microsystems). Excitation and emission wavelengths for Cy3 were 433 and 560–618 nm respectively; Alexa488-labeled excitation wavelength was 488 nm and its emission at 510–570 nm. Serial 0.2 μm scans were obtained in the Z-plane.

For the quantification of dual-labelling, we used a 20x lens. Image J software combined with loci. tools plugin (BIO-FORMATS, University of Wisconsin-Madison) was used to count the number of positively labelled cells in 20-32 Z-plane sections from control and shIRS1 groups of both females and males. For IRS1+EGFP dual-labelling quantifications, data were expressed as the percentage of double-labelled neurons (IRS1+AAV), divided by the total number of AAV-positive neurons. 3 AAV controls and 4 AAV shIRS1 females and 7 AAV controls and 7 shIRS1 males were analyzed by a researcher blind to the condition (2 dentate gyri per animal). For CR+EGFP, PV+EGFP, and SOM+EGFP dual-labelling quantifications, data were expressed as the percentage of double-labelled neurons (neuronal marker + EGFP), divided by the total number of neuronal marker-positive cells. 4 AAV shIRS1 females and 7 AAV shIRS1 males were analyzed by a researcher blind to the condition (1 dentate gyrus per animal).

For quantification of synaptophysin signal in AAV infected neurons, we used a 63x lens. Image J software combined with loci. tool plugin was used to count the number of synaptophysin points (minimum 3 pixels were considered) in one AAV-positive fiber in 10-15 Z-plane sections from control and shIRS1 groups. Three AAV controls and four AAV shIRS1 female animals, 10 different axons per animal were analyzed by a researcher blind to the condition. Data are calculated as the number of synaptophysin positive signals on the AAV-infected fiber, normalized to the area of the fiber.

The experimental outcomes from these procedures were, IRS1 mRNA expression, done in transfected cells. Behavioural changes in rats inoculated with the virus. And postmortem analysis of AAV tracing, colocalization quantification studies, and synaptophysin quantification.

Statistical analysis

The analysis was carried out with Graph Pad software (GraphPad Prism V8 software, GraphPad, La Jolla, CA, USA). Data reported as the mean \pm SEM and the “*n*” the number of independent subjects. Data were subjected to Kolmogorov-Smirnov test for Gaussian distribution. If normality was confirmed, one-tailed or two-tailed Student’s t-test with probability set at $\alpha < 0.05$ was used, otherwise the non-parametric Mann-Whitney *T* test was applied as indicated. Pearson test was used for correlation analysis. The correlation coefficient *r* values (± 0.1 to ± 0.3) reveal weak, values around (± 0.3 to ± 0.5) reveal medium and higher than ± 0.5 reveal a strong correlation. Positive or negative *r* values mean positive or negative correlations, respectively.

5.3. Results

5.3.1. shRNA IRS1 knocks down IRS1 mRNA levels in HEK293 cells

To verify the knockdown efficiency of the shRNA against IRS1, we measured IRS1 and IRS2 mRNA expression in transfected HEK293. We used two constructs, expressing two different shRNA sequences (shIRS1A and shIRS1B) compared to plasmid control. We observed that expression of IRS1 but not IRS2 mRNA was significantly reduced by both sequences (Fig S5.2). The shRNAs were designed against rat, but they displayed 98% homology with humans, so we could translate the results from human cells to rat IRS1.

5.3.2. AAV-shIRS1-EGFP injection in dorsal hippocampus alters spatial memory, especially in females, but not social memory, nor anxiety or depressive symptoms

AAV-shIRS1 and AAV control (Fig 5.1A) were generated in HEK293 cells with a titration of $1.02\text{E}+11$ GC/mL and $4.94\text{E}+11$ GC/mL, respectively. AAV particles were bilaterally injected by stereotaxis in the dorsal HPC (AP -4.36 mm, \pm ML 2.5 mm, DV -2.5 mm from Bregma) (Fig 5.1B). Control and shIRS1 injected animals were subjected to behavioural paradigms one month after viral particle injection (Fig 5.1C). Both paradigms evaluate the memory in rodents exploiting their innate exploratory preference for novel stimuli, the NOR test examines the capability of the animal to remember a familiar object. To quantify it, the DI is obtained by subtracting the time exploring the familiar object to the time exploring the novel one; divided by total exploring time. We observed that the shIRS1 injected females animals (DI; 0.24 ± 0.13) discriminate both objects significantly less than the AAV control injected animals (DI; 0.56 ± 0.11); whereas we did not see the same effect in males receiving shIRS1 (DI; 0.44 ± 0.05), compared to controls (DI; 0.47 ± 0.06) (Fig 5.2A). The data are represented by mean \pm SEM and analyzed using unpaired Student's t-test (* $p < 0.05$).

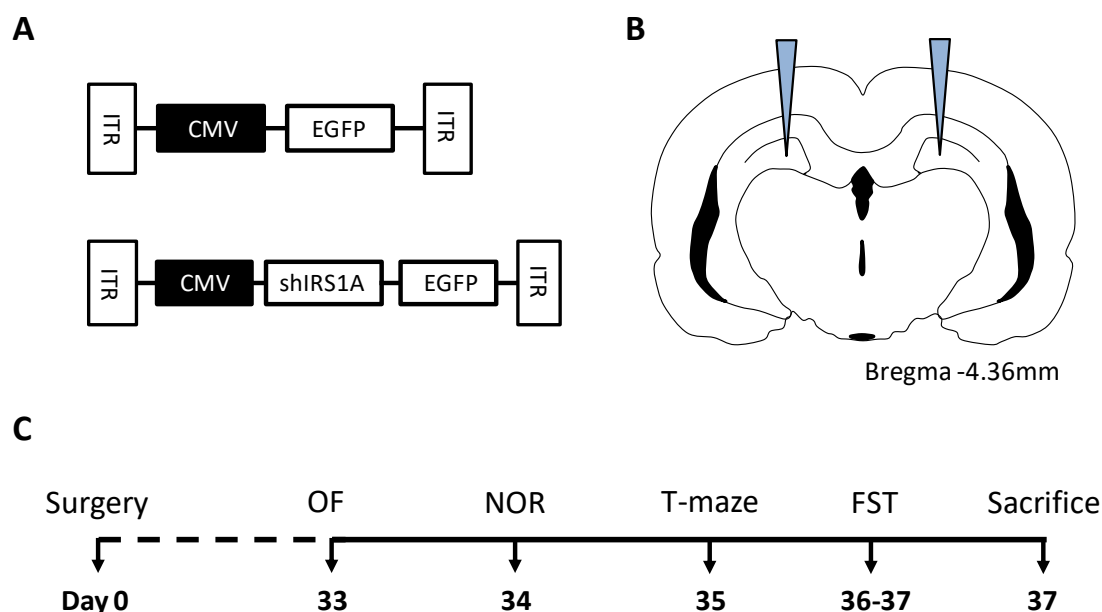


Fig. 5.1. Experiment design. Bilateral injection of the viral particle (AAV-CMV-GFP, $4.94\text{E} + 11$ GC/mL or AAV-CMV-shIRS1-EGFP, $1.02\text{E} + 10$ GC/mL) (A) into the dorsal hippocampus (AP -4.36 mm, ML ± 2.5 mm, DV -2.5 mm from bregma); 2 μL of viral particle per hemisphere were injected (B). Behavioural tests started 33 days post-injection, and the animals were sacrificed at day 37 (C).

In the T-maze test, we recorded the number of entries and the time exploring the novel arm. Females in the shIRS1 group showed reduced DI (-0.03 ± 0.10) in the time spent in novel arms compared to controls DI (0.23 ± 0.08) (Fig 5.2B). Similarly, in males, shIRS1-injected male rats

had significantly lower DI value (-0.00 ± 0.04) than controls (DI; 0.16 ± 0.07) in the time spent in the novel arm. Females showed significantly lower DI (0.02 ± 0.02) in the number of entries (Fig 5.2C) to the novel arms compared to the control group DI (0.14 ± 0.06). Also, in males, the number of entries to the novel arm was lower (DI; -0.02 ± 0.03) than controls (DI; 0.09 ± 0.04). These results suggested that the downregulation of IRS1 impairs the memory of the familiar arm, both in female and male rats. We evaluated anxiety by the latency to cross for the first time the open field and observed lower but not significantly different shIRS1 (43.13 ± 11.06) compared to control AAV (61.67 ± 22.48) injected females. Nor differences either in shIRS1 (14.50 ± 4.59) and control AAV (25.86 ± 5.82) injected males (Fig 5.2D). Finally, we assessed depressive symptoms in the animals by the forced swimming test. As above, hippocampal injection of shIRS1 had no effect, since immobile time was equal between groups, control (111.20 ± 9.15) and shIRS1 (140.20 ± 19.21) injected female rats and control (157.80 ± 15.17) and shIRS1 (184.90 ± 7.18) injected male rats (Fig 5.2E). Swimming time and climbing were not different either between groups (data not shown).

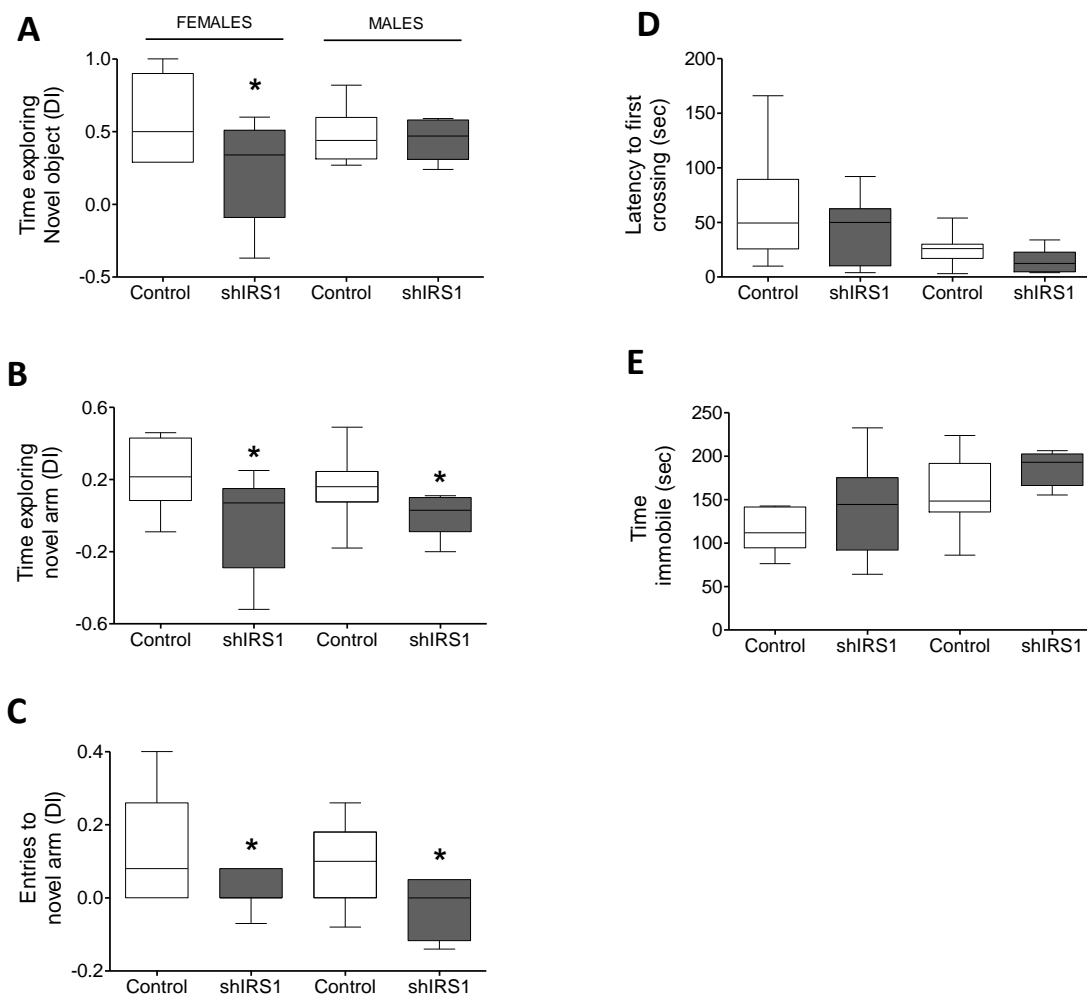


Fig. 5.2. AAV shIRS1 impairs spatial memory. Time exploring the novel object in the Novel Object Recognition test (A). Time exploring the novel arm (B) and the number of entries to the novel arm (C) in the T-maze test. Latency to cross the center of the arena for the first time in the Open Field test (D). Time spent immobile in the Forced Swim test (E). Data are expressed as discrimination index [(Time exploring novel – time exploring familiar)/total time exploring], presented as mean \pm whiskers at maximum and minimum ($n = 7-8$ per condition) and analyzed using one-tailed Student t test * $p < 0.05$.

5.3.3. AAV DJ8 tracks dorsal hippocampus efferent connections

Dorsal HPC efferent projections reach MS and MM through the fimbria-fornix white track (Fig 5.3A). We inoculated AAV-CMV-shIRS1-EGFP and AAV-CMV-EGFP in the dorsal HPC. We observed fibers and cells in the DS, an area within the hippocampal formation (Fig 5.3B), and in the polymorphic area or *hilus* of the dentate gyrus (PoDG) (Fig 5.3C). As expected, we found fibers in the fornix (f) (Fig 5.3D) and consequently in MS (Fig 5.3E) and MM, specifically in the SuM (Fig 5.3F).

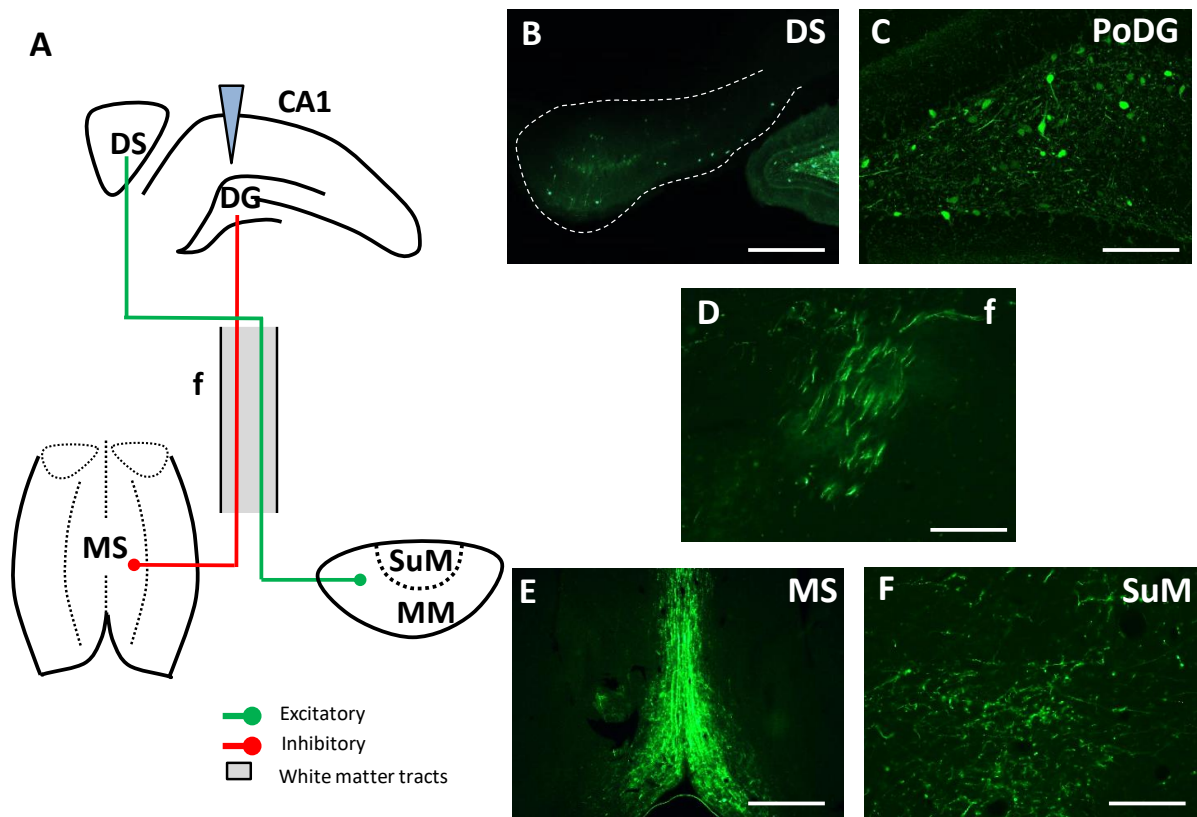


Fig. 5.3. AAV DJ8 traces dorsal hippocampus efferents. Schematic representation of the projections from the dorsal hippocampus, where the viral particle was inoculated (A). Representative images of 3 animals (per condition) and 3 slices each of neuronal somas in the dorsal subiculum (DS) (B), and the polymorphic layer of the dentate gyrus (PoDG) (C); AAV-labeled fibers in the medial septum (MS) (E) and supramammillary nucleus (SuM) (F) via fornix (f) (D). Scale bar = 200 μ m (B). Scale bar = 100 μ m (C-F).

5.3.4. AAV-DJ8 labels dorsal specific hippocampal neurons

AAV infected mostly neurons in the *hilus* of the DG, but not the granule layer (Fig 5.4). To a lesser extent, the AAV entered the CA1 (Fig S5.3). We observed that astrocytes were not infected by the AAV tropism (Fig S5.4). Diverse GABAergic cell types localize in the DG, so we performed immunostaining of neuronal markers to characterized AAV-infected neurons. We found that AAV labels co-localized with CR (Fig 5.4C), PV (Fig 5.4D), and SOM (Fig 5.4E) neurons. The quantification of the EGFP-cell marker double labelling revealed that $48.78\% \pm 2.97$ of SOM cells and $42.88\% \pm 3.75$ of PV had been infected with AAV, whereas the number of infected CR cells was slightly lower; $32.30\% \pm 3.39$, suggesting that the SOM and PV neuronal activity has been more affected by IRS1 silencing (Fig 5.4B).

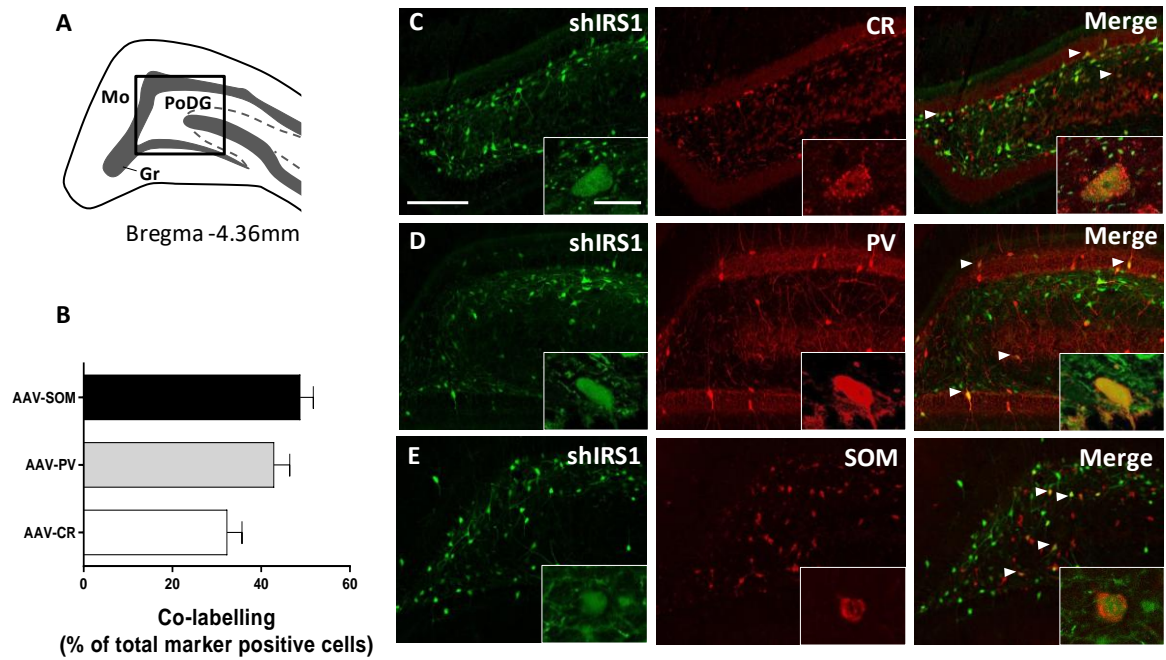


Fig. 5.4. AAV DJ8 labels dorsal specific hippocampal neurons. Schematic representation of the dentate gyrus of the dorsal hippocampus (A). Percentage of double-labelled neurons (EGFP + neuronal marker), relative to the total number of neuronal marker-positive cells. Data are represented as the mean \pm SEM ($n = 11$) (B). Representative images of 4–6 animals (per condition) and 3 slices each of EGFP (green) co-localizing with calretinin (CR) (C); Parvalbumin (PV) (D); and Somatostatin (SOM) (E) positive neurons in the polymorphic area (PoDG) of the dentate gyrus. Scale bar = 100 μ m. Scale bar = 20 μ m (insets).

5.3.5. AAV DJ8 axons in the medial septum reach PV and ChAT occupied areas

We observed that MS was strongly innervated by AAV-labelled axons. The fibers seem to be restricted to the most medial part. To evaluate the type of neurons potentially regulated by the AAV-labelled projections, we carried out a series of immunolabelling analysis. We observed that AAV projections co-localized mostly within PV (Fig 5.5B) and ChAT (Fig 5.5C) areas. Also, sparse CB positive neurons fall within the axon labelled areas (Fig 5.5D). The field where CR neurons appear is completely devoid of EGFP fibres (Fig 5.5E).

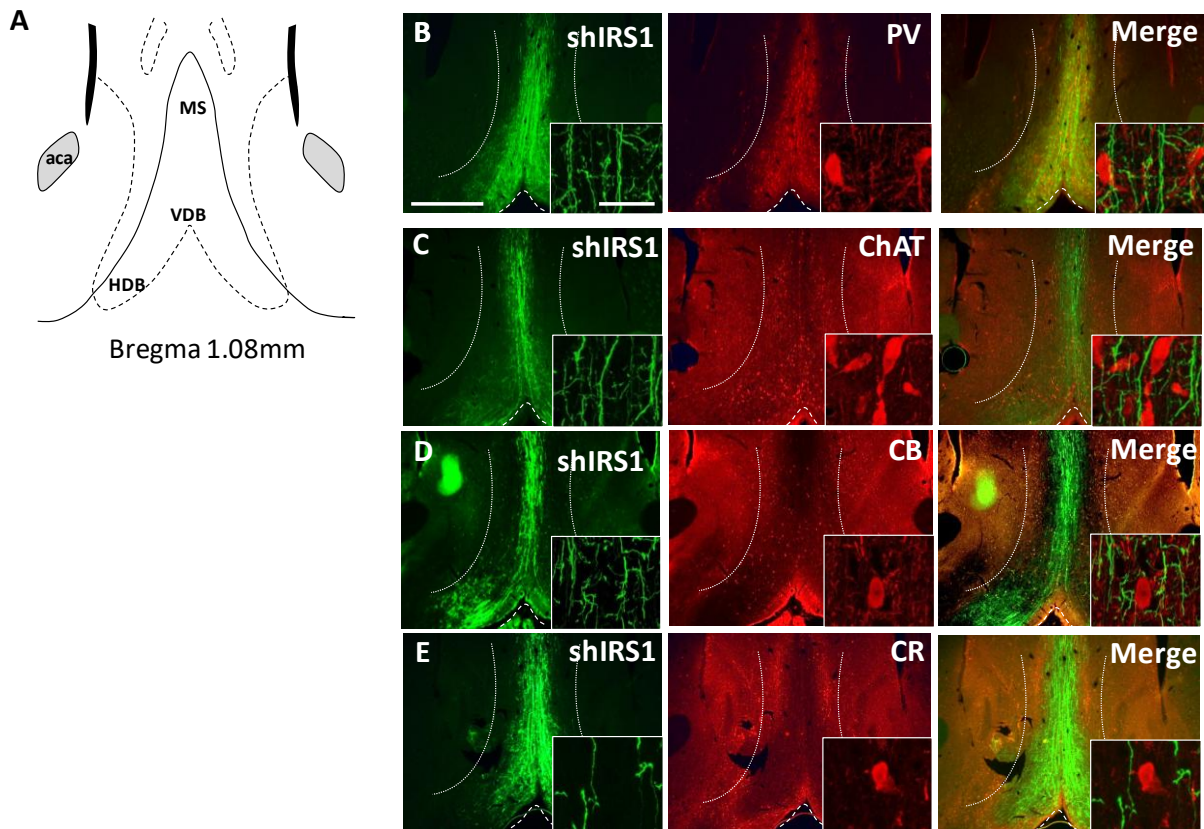


Fig. 5.5. AAV-labeled axons in the medial septum. Schematic representation of the medial septum (MS), vertical limb diagonal band (VDB), and horizontal limb diagonal band (HDB) (A). Representative images of 3 animals (per condition) and 3 slices each of showing AAV-infected neuron projections co-localized with parvalbumin (PV) (B), cholinergic (ChAT) (C) and calbindin (CB) area (D). We observed no co-localization of AAV green fibers with calretinin (CR) positive neurons field (E). Scale bar = 100 μ m. Scale bar = 20 μ m (insets).

5.3.6. AAV-shIRS1 results in lower expression of IRS1 in the dorsal hippocampus and correlates with behaviour

To ascertain if AAV-shIRS1 corresponded to reduced IRS1 protein expression, double IRS1 immunofluorescence and EGFP expression were quantified and expressed in relation to total EGFP-positive neurons (Fig 5.6B). We observed that the AAV-shIRS1 resulted in a reduced number of double IRS1-EGFP neurons ($67.88\% \pm 1.22$), compared to control AAV ($80.28\% \pm 1.9$). This result is indicative of a knockdown of IRS1 expression in the dentate gyrus where the AAV-shIRS1 was inoculated.

Moreover, to elucidate if the different degrees of reduced expression could account for the behavioural variability, we performed linear regression and applied the Pearson test, correlating the DI in time spent in the novel arms (T-maze) with the degree of IRS1 reduction (as shown in Fig 5.6B). We observed that the correlation between DI time in novel arms in the T-maze, was positive (Correlation DI time with shIRS1 $r = 0.63$, and DI time vs control ($r = -0.15$) (Fig S5.5). These results suggest that in AAV-shIRS1 subjects the degree of IRS1 reduction correlates better with the behavioural impairment than in control subjects.

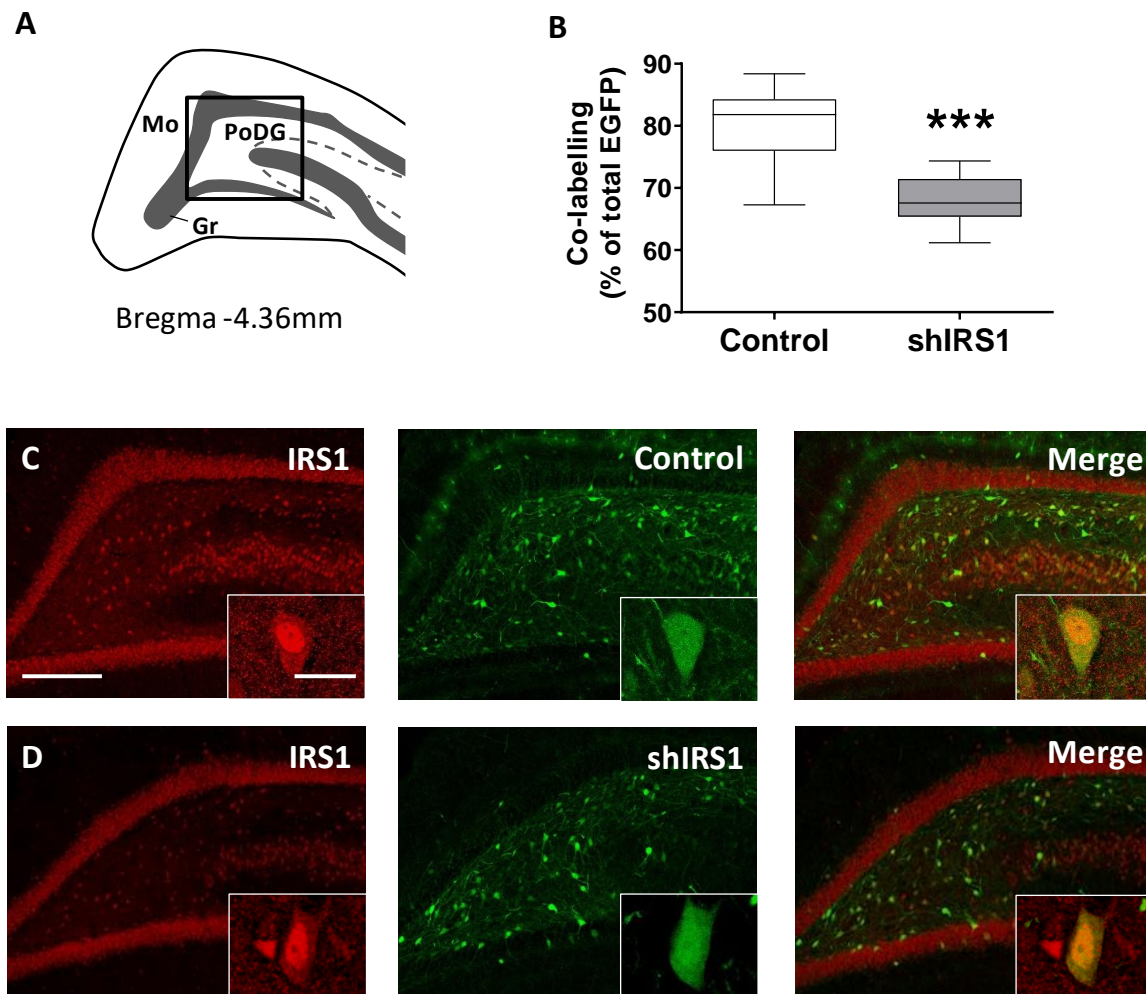


Fig. 5.6. Silencing effect of shIRS1 in the dorsal hippocampus. Schematic representation of the dentate gyrus of the dorsal hippocampus (A). Percentage of double-labeled neurons (IRS1 + EGFP), relative to the total number of EGFP-positive neurons (B). Representative images of IRS1, EGFP and co-localization of both labelling (merge) in the polymorphic area (PoDG) of control (C) and shIRS1 (D) animals. Data are represented as the mean \pm SEM (n = 10–11). Data were analyzed using one-tailed Student t test *** $p < 0.0001$. Scale bar = 100 μ m. Scale bar = 20 μ m (insets).

5.3.7. AAVshIRS1 infection results in lower synaptophysin positive labels compared to AAV control

To explain the possible mechanism underlying the observed behavioural results we hypothesized that the reduction in IRS1 signalling could impair synaptic plasticity, based on previous data where insulin resistance reduced synaptophysin expression^[340] and reduced plasticity^[341,342]. To evaluate the effect of shIRS1 in axons in the MS area (Fig 5.7A), we quantified synaptophysin labelling normalized by the green-labelled area. We observed that the number of synaptophysin-positive labels in axons was significantly lower in AAV-CMV-shIRS1-EGFP axons (307.90 \pm 20.40) than in controls AAV-CMV-EGFP (444.30 \pm 27.13) (Fig 5.7B). These results suggest that knocking down IRS1 reduces synaptic plasticity, as measured by synaptic vesicle proteins, used as an indirect marker of synaptic activity. Representative images are shown for AAV control (Fig 5.7C) and AAV-shIRS1 (Fig 5.7D).

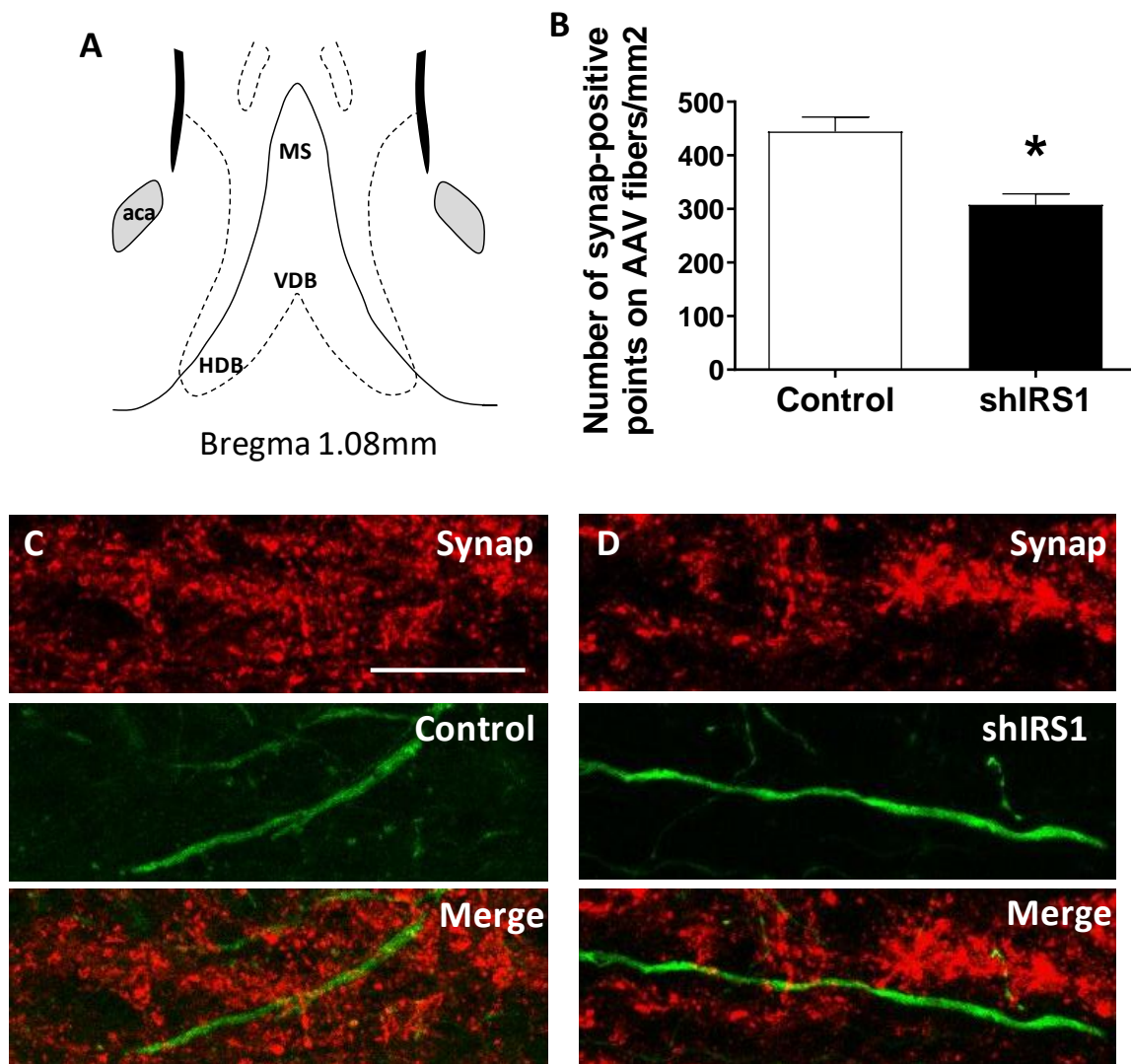


Fig. 5.7. AAV-CMV-shIRS1-EGFP inoculation in the dorsal hippocampus results in lower synaptophysin positive labels in fibers reaching medial septum. Schematic representation of the medial septum (A). The number of synaptophysin (Synap) positive signals on the EGFP-labeled fibers (B). Representative images of synaptophysin co-localization with AAV control (C) and EGFP-shIRS1 (D) fibers. Data are represented as the mean \pm SEM of the number of synaptophysin contacts with the green AAV labelling per mm² of the fiber (n = 3–4; 10 fibers per animal). Data were analyzed using one-tailed Mann Whitney t test * $p < 0.05$. Scale bar = 10 μ m.

5.4. Discussion

In our study, we show that infection of shIRS1 using an AAV2/DJ8 derived virus inoculated in the hippocampal area affects hippocampal memory formation in female and male rats. It does not alter anxiety parameters in any sex. Loss of spatial memory is one of the first symptoms in AD, caused by progressive loss of neuronal circuitries. Diabetes, dyslipidemia, and cardiovascular factors have been demonstrated to play a role in degenerative diseases like AD and frontotemporal dementias^[343]. These pathologies are linked to brain insulin resistance, which triggers cognitive decline and AD^[344]. Most research addressing insulin resistance in the brain assumes that neuroinflammation causes insulin resistance, and this, in turn, aggravates neuronal damage, stimulating the neuroinflammatory process. Although neuroinflammation is a general process affecting the whole brain, in AD patients one of the first clinical symptoms is the loss of spatial memory. Thus, it is likely that only particular circuitries involved in this memory are affected at the early stages of the disease before massive neuronal death occurs. Major hippocampal circuitries like the septo-hippocampal connections^[181] and the circuit of Papez^[187] are crucial for hippocampal-dependent memory formation.

In this study, we made use of an AAV carrying a shRNA targeting IRS1. We confirmed mRNA downregulation by PCR *in vitro* studies and reduced protein expression *in vivo* by immunofluorescence. We also confirmed that the shIRS1 did not affect significantly IRS2 expression, so the knocking down is specific for this isoform. Most studies in the literature have focused on the IRS1 inactivation due to serine phosphorylation. Serine residues are phosphorylated by inflammatory signals. In this study we have used shIRS1 to reduce the expression of IRS1, thus IRS1 signalling activity is reduced. This study is not a knockout, so a residual activity is present, and there are no compensations either which may alter the specific effect of an IRS1 reduced expression. Importantly, we wanted to isolate the IRS1 effect, since inflammatory processes affect several pathways, one of the IRS1, we aimed to examine the IRS1 contribution in discrete neuronal pathways in the absence of neuroinflammation. This may indicate a way to improve brain health in human pathologies where IRS1 signalling is affected.

Females rats inoculated with the AAV-CMV-shIRS1-EGFP performed significantly worse in the NOR test, compared to controls inoculated with AAV-CMV-EGFP. The NOR test evaluates the subject memory by measuring the recognition of familiar objects. When the animal does not remember having explored an object, it will explore both equally, as they are both equally new to the subject. Unlike female subjects, males inoculated with shIRS1 remember the familiar object, and they behaved similarly to control injected males. This result suggests that the female brain may display a higher susceptibility to insulin resistance, this would agree with the observation that AD is more prevalent amongst females^[345]. In humans, AD is reported to be more prevalent in women than in men^[346]. Reports indicate that around 70% of AD patients are women in the United States^[347,348] and in Europe^[349]. Several hypotheses have been proposed to explain that fact. The most evident is that women have a longer life span than men, but even after normalizing to it, other biological factors have been considered. Amongst others, female higher sensitivity to the presence of one APOE 4 allele has been proposed^[350,351], since this isoform increases the probability to suffer from AD^[352]. APOE is the major lipid transporting molecule in the brain and the isoform APOE 4 has less capacity to shuttle lipids than the others, thus, given the importance of cholesterol in neuronal function^[353], cholesterol dysregulation is expected to contribute to degenerative pathologies^[354]. Because dyslipidemia and insulin resistance act in a vicious cycle to promote pathologic processes contributing to degenerative diseases; under the light of our results, we proposed that females, even with normal estrogens levels (in this study female rats were not ovariectomized) may be more susceptible to insulin resistance (due to reduced IRS1 function) than male. We hypothesize that this could be a major contributing factor to the higher prevalence of AD in women; however, further studies are required to unveil the underlying mechanism to this effect.

The T-maze evaluates the spatial navigation capability. Exploration of novel spaces is a hallmark of rodent, and as expected, female and male subjects inoculated with control AAV explored the new arm longer time than the familiar one. Both females and males injected with shIRS1 failed to discriminate between the maze novel and familiar arms, indicating that they do not

remember the arm previously visited. These results agree with former studies reporting that IR silencing using a lentiviral derived particle inoculated into the HPC impaired spatial memory^[355]. Importantly, in this task, we found no difference between male and female subjects. One possible explanation for this result is that the T-maze can be considered a more difficult task than the NOR task, it also measures navigation and NOR evaluates recognition. Therefore, we hypothesize that the NOR test may more sensitive to smaller neurological deficiencies^[118].

We observed no differences in anxiety-like behaviours. This may be in contrast with previous reports indicating that hippocampus-septal connections may regulate anxiety^[356,357]. Because the circuit targeted with the AAV DJ8 virus has been proved very specific, we consider that we have only affected the hippocampal processing of spatial memories.

In this study, AAV DJ8 inoculated in the hippocampal area is mostly found in neurons within the PoDG or *hilus* of the DG and to a lesser extent in the DS. Sparse neurons were found in the CA1 regions (Fig S5.3). We observed abundant fibers via the fimbria-fornix pathway. This pathway is a white matter tract that contains hippocampal efferents (to MS and MM) and afferents (from septal cholinergic projections^[358]). Consistent with hippocampal efferent we observed abundant fibers through the fornix. Following Papez circuitry, we also found fibers in the MM. Consistently, no thalamic staining was observed (data not shown). In addition, we found extensive green fibers in the MS. Traditionally, MS has been considered to receive projections from CA1 GABAergic SOM neurons^[359]. However, long-range projection interneurons have been localized in the DG *hilus* innervating the MS^[360]. Recently, these DG long-range projection interneurons have been characterized as a new type of SOM interneuron and named as *hilar interneurons with long axons* (HIL). HIL neurons co-exist with hilar SOM interneurons (the SOM HIPP neurons)^[180]. Long axon SOM neurons provide regulation to both nearby PV interneurons and display collaterals projecting to the MS, where they contact ChAT and PV neurons^[180].

We cannot rule out the effect of IRS1 silencing in glutamatergic neurons in the HPC. However, since we have seen a high proportion of AVV infection in the *hilus* of the dentate gyrus, where most neurons are GABAergic, for clarity, we decided to focus this study on the GABAergic connections. This rationale is backed up by the strong green labelling we observed in the PV area within the MS, which corresponds to hippocampal SOM neurons projecting to the septal area. This, by no means, suggests that it is the only mechanism underlying the IRS role in neuronal activity. To better understand the role of IRS1 in Glutamatergic, different AAV serotypes may improve infection in the glutamatergic type of neurons.

We immunocharacterized the neurons within the DG infected with AAVDJ8 and confirmed the co-labelling with the SOM antibody. Moreover, AAV fibers were found where the PV and ChAT neurons are in the MS^[180], therefore, we assume that our AAV can be affecting both types of hilar SOM, the short and long projecting neurons. Both types of hilar SOM interneurons play an important role in regulating hippocampal function. The DG is the first phase of the classical trisynaptic circuit^[361,362], defined as the circuit starting from the EC to the CA3 pyramidal neurons, and from these to the CA1. This circuit is crucial for the formation of new memories^[363,364]. Thus, information coming from the entorhinal is regulated with precise timing by the strong inhibitions within the DG, this modulates the output of granule cells to CA3, where spatial memory forms a spatial pattern. HIPP provides dendritic inhibition controlling the entrance of spatial information from the EC to the DG. In addition, the inhibition mediated by HIL cells is proposed to contribute to the temporal coordination of MS and local rhythmic DG activity^[180]. Since we do not see soma labeling in the MS, we assume that AAV only travels anterogradely through the fimbria-fornix to MS, therefore, we assume that MS neuronal activity is affected via hilar SOM long projections.

We found that axons from the HPC innervating MS (the long projecting SOM) have reduced synaptophysin labelling, indicative of reduced synaptic plasticity. Even though we cannot rule out other mechanisms, this result illustrates that the reduction in IRS1 function impairs long projecting SOM neuronal plasticity with behavioural consequences. Synaptophysin labelling is a marker of presynaptic activity; together with postsynaptic markers it is considered a measure of the circuit

synaptic plasticity. Presynaptic alterations of synaptophysin labelling have been reported as an early event in AD^[365].

We speculated that IRS1 silencing may alter neuronal presynaptic activity, and even if we cannot rule out an alteration at the postsynaptic level, we focused on the connection hippocampus-septum, and we used synaptophysin labelling as a presynaptic marker of synaptic activity. Indeed, we found that synaptophysin staining was reduced in shIRS1 infected neurons compared to controls. The mechanism underlying this observation can be a reduced IRS1-PI3-AKT pathway, that regulates GLUT4 translocation to the membrane^[366], which would hamper glucose availability at the nerve terminals, thus impairing synaptic activity^[367]. The long-term modulation of synaptic activity is achieved (amongst others) by regulating the expression of several neurotransmitter vesicle proteins. This has been shown with a high-fat-diet model that reduced IGF-1 and synaptophysin levels. These were recovered after IGF-1 signalling activation^[368]. Also, BDNF has been shown to modulate synaptic plasticity by regulating synaptophysin expression^[369,370].

IRS1 has been mostly studied in the context of insulin and IGF-1 signalling, however, given their scaffolding function it is plausible that IRS1 mediate several neurotrophic factors, including BDNF^[328]. This would agree with the fact that even a small reduction in the IRS1 expression function has resulted in a behavioural defect.

In addition to SOM, we found co-labelling with GABAergic PV interneurons, and to a lesser extent with CR neurons. Fast spiking PV interneurons regulate network oscillation and synchrony^[371]. Previous reports demonstrate that PV dysregulation in the DG affects memory and anxiety^[372]. However, in our present work, we do not see alterations in anxiety nor social behaviour or depressive symptoms, but specifically spatial memory.

Under the light of our results, we hypothesized that IRS1 signalling is required for both SOM and PV inhibitory action, by reducing synaptic plasticity. We believe that we have affected two pathways by the AAV infection; on one hand, there may be disinhibition of the information coming from the EC, hampering the formation of a clear spatial pattern in CA1. On the other hand, there may be disinhibition of PV and ChAT neurons in the MS, which in turn may increase PV and cholinergic action back into the HPC, this dysregulation can thus impair the excitatory/inhibitory balance.

Moreover, the Papez circuitry is essential to memory formation^[373]. Papez circuitry starts in the subiculum, a part of the hippocampal formation. Evidence supports that the sequential pathway from the subiculum to the MM via the fornix, and from there to the anterior thalamus, is required for spatial memory in rats^[374] and humans^[375]. Moreover, this circuitry is found severely affected in AD patients^[376]. Because viruses do not “jump” to adjacent neurons, the effect of the IRS1 silencing is confined to the hippocampal projection to the MM via the fornix. Some reports that have specifically disrupted this efferent projection indicate that it has only a mild effect on memory formation^[377]. Other studies, while emphasizing MM function in memory formation^[378], indicate that other projections from brain stem nucleus (e.g. the tegmental nuclei of Gudden), and not the hippocampal formation, are critical for sustaining MM function^[378,379]. Therefore, taking together these findings, we may assume that the effect we have observed disrupting memory formation in rats inoculated with AAV shIRS1 is most likely due to IRS1 knockdown in hilar DG GABAergic neurons, modulating the incoming flow of information from the EC and projections to the MS.

Taking together all these findings, our works supports that IRS1 signalling in GABAergic hippocampal neurons, both interneurons, and MS projecting neurons is required for forming spatial memories. In this model, we mimic insulin resistance by silencing IRS1 in an otherwise healthy brain (i.e no inflammatory ambiance). In neurodegenerative diseases, inflammatory conditions induce insulin resistance, and concomitantly, loss of cognitive function. Our results indicate that insulin resistance, in the absence of inflammatory conditions, is sufficient to cause memory impairments, suggesting that this could be at the very onset of neurodegenerative diseases.

6

Chapter 4

TARGETING NEUROINFLAMMATION IN A
MICE MODEL OF ADHD WITH ALTERED PAIN
SENSITIVITY. A PILOT STUDY

6.1. Introduction

ADHD is one of the most common neurodevelopmental disorders in children and adolescent worldwide (incidence 8-12%)^[380]. Main symptoms are impulsivity, hyperactivity, and problems with sustained attention^[381]. ADHD often coexist with other neurodevelopmental alterations (i.e. autism spectrum disorder); and also with anxiety and depression^[381]. Currently, pharmacological treatment implies psychostimulants chronic administration (e.g. methylphenidate) which raises reasonable concerns regarding its potential dangerous long-term effects^[382,383] including addiction^[384], and anxiety^[385]. Therefore, it is of fundamental interest to investigate the etiology of the disease to develop new therapeutic strategies. Strong evidence has indicated that dopaminergic system dysfunction stands at the onset of ADHD^[386]. To investigate the pathophysiology of ADHD, several animal models have been developed^[387]. Among them, neonatal lesion with 6-hydroxydopamine (6-OHDA) has prevailed since it is specifically toxic to central dopaminergic pathways. This model simulates key hallmarks of the human disease including hyperactivity and its paradoxical response to psychostimulants^[388,389].

Increased inflammation during early neurodevelopment is suspected as a risk factor aggravating and/or triggering ADHD symptoms^[390], although no mechanism has been proposed, yet. Epidemiological studies including meta-analyses reveal that patients with ADHD are more likely to suffer asthma, allergic rhinitis, atopic dermatitis, and allergic conjunctivitis (well-known inflammatory conditions) than control subjects^[391-393]. Moreover, maternal inflammatory status can prompt the occurrence of ADHD in the offspring; one prospective study (more than 23000 patients) revealed that a maternal history of autoimmune disease was associated with an increased risk of ADHD^[394]. Yet, studies evaluating serum inflammatory markers in patients have not reached strong conclusions^[395]. Other studies have shown certain IL-6 and TNF α gene polymorphisms associated to ADHD patients^[396]. Furthermore, it has been found that oxidative stress markers levels, such as malondialdehyde (MDA) and nitric oxide (NO), were increased in ADHD children^[397] and adults^[398,399]. That neuroinflammation biomarkers in ADHD children were associated to comorbid anxiety, not to “pure” ADHD^[400]. For this subset of patients, environmental stress (e.g. confinement) will aggravate ADHD symptoms. Thus, is urgent to elucidate the relationship between neuroinflammation and comorbidities in ADHD patients.

Given the previous work we have carried out with ABA, where we have shown that oral ABA administration can ameliorate neuroinflammation-induced brain alterations, we proposed that ABA could be a good candidate to treat psychiatric conditions of neuroinflammatory etiology. Furthermore, in human SH-SY5Y cell culture, ABA protected DA neuroblastoma cells from damage and death induced by 6-OHDA neurotoxicity^[401].

Attention and pain are intimately related; thus, attention modulates pain perception as demonstrated by studies where subjects engaged in a task requiring attention perceived less pain to same stimulus than subjects not doing such task^[402]. Conversely, chronic pain reduces attention span^[403]. In humans, recent evidence indicates that ADHD increases pain perception in adults^[404,405] and the prevalence of generalized pain is higher in ADHD patients (up to 80%) compared to control population (17%)^[406]. Interestingly, methylphenidate treatment for ADHD can partially reduce nociception, in adults^[407]. Also, children with debilitating chronic pain present higher risk of having comorbid neurodevelopmental disorders, including autistic traits and symptoms consistent with ADHD^[408].

Interestingly, neuroanatomical studies confirm that attentional processes and pain transmission use identical neural networks. Thus, it is plausible that neuroinflammation affecting synaptic function in specific brain areas, is a risk factor for ADHD symptoms and for the altered pain sensitivity.

The available data shows the importance of neuroinflammatory mechanisms in both altered pain sensitivity and ADHD. This pilot study represents preliminary data aiming to elucidate whether ABA treatment in a preclinical model of ADHD can ameliorate ADHD symptoms and

altered pain sensitivity. We hypothesized that this effect would be due to reduced inflammation within relevant brain areas, such as ACC-insula.

6.2. Materials and methods

The workflow is depicted in Fig. 6.1.

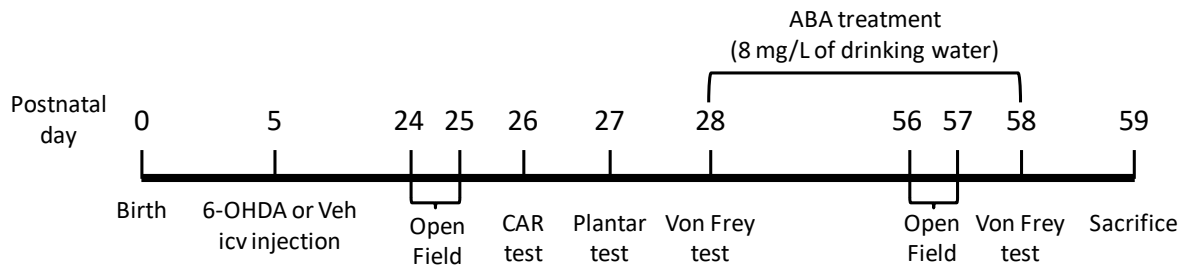


Fig. 6.1. Schedule of the experiment design. 6-OHDA icv (intracerebroventricular) injection was performed at P5. ABA administration (8 mg/L in the drinking water) started in P28 and finished in P58. Behavioral tests were carried out between P24-P28 and two paradigms (Open field and Von Frey test) were repeated after 4 weeks of ABA treatment. Animals were sacrificed at P59.

Animals and surgical procedures

Eighteen C57BL/6 mice (9 females and 9 males) were bred and kept in the animal facility of Centre Paul Broca-Nouvelle Aquitaine (University of Bordeaux). The procedures followed directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes. The experiments were approved by the Ethics Committee of the University of Bordeaux (approval number APAFIS-27681). Once the mice were born, they were housed with their mothers and maintained on a 12 h:12 h light–dark cycle (lights on at 7 am) with food and water *ad libitum*.

At postnatal day 5 (P5), pups received an icv injection of 6-OHDA hydrobromide (Sigma-Aldrich, France) or vehicle as described in Bouchatta et al. 2018^[388]. Thirty minutes before surgery, mice were injected with desipramine hydrochloride pretreatment (20 mg/kg sc; Sigma-Aldrich, France), an inhibitor of the noradrenergic re-uptake, to protect noradrenergic system against the 6-OHDA-induced degeneration. Mice were anesthetized with isoflurane 4% and maintained with isoflurane 2% during surgery. Stereotaxic coordinates used to infuse 25 µg of 6-OHDA dissolved in 3 µl ascorbic acid 0.1% or vehicle into one of the lateral ventricles were AP -2 mm, ±ML 0.6 mm, DV -1.3 mm from bregma. After weaning (P21), mice were divided per sex in groups of 2-4 animals to reduce stress due to social isolation.

Abscisic acid treatment

At P28, 6-OHDA mice were divided randomly into two experimental groups per sex: animals that received ABA (Sigma-Aldrich, Spain) in the drinking water (8 mg/L) and animals that received vehicle (VEH). Due to the limited number of animals, the Sham group of both sexes received VEH in the drinking water. All groups were fed *ad libitum* for 4 weeks. The body weight and water intake were monitored once a week per cage.

Behavioural procedures

All the behavioural paradigms were carried out at P24. Open field and Von Frey test were repeated at the end of ABA treatment (P56-58). One week before the start of the behavioural testing, daily handling to all the animals was carried out. In all the procedures, mice were habituated to the testing room 30 minutes before performing each behavioural paradigm.

Behavioural tests were conducted during the day with a dim light and at the same time zone to avoid activity differences caused by the light-dark cycle.

Open Field; The open field apparatus consisted of a grey PVC box (40 x 40 x 30 cm) combined with infrared sensors. Mice were placed in the centre of the open field and allowed to freely explore the arena for 10 min. The test was divided in two days to reduce the effect of excitation caused by novelty on the spontaneous locomotor activity. The box was cleaned with a 30 % ethanol solution between animals to eliminate olfactory cues. The velocity (cm/s) and the distance travelled (cm) were quantified. Animals were recorded using the EthoVision XT video tracking system (Noldus, Wageningen, Netherlands).

Cliff avoidance reaction (CAR) test; CAR assessment was performed placing mice over a round plastic platform (20 cm diameter, 50 cm height). Animals were gently placed over the paradigm with the forelimbs close to the edge and the latency to the first fall was registered. Then, mice were immediately placed back on the platform and the number of falls was recorded for 60 minutes. If one animal did not fall, it was not removed from the platform during the same duration of time. The paradigm was cleaned with a 30 % ethanol solution between animals. Usually, animals tend to avoid falling from a high surface, thus when a mouse fell from the platform it is showing an impaired CAR. Animals were recorded using the MediaRecorder video software (Noldus, Wageningen, Netherlands).

Plantar test (Hargreaves apparatus); The thermal nociceptive response was assessed using the plantar test paradigm (Ugo Basile, Varese, Italy). Each mouse was placed inside individual cages (10 x 10 x 14 cm) with a glass pane floor where animals could freely move. During the first 30 minutes, mice were habituated to the environment. Using an infrared (IRed) generator, ventral surface of left and right hind paws was stimulated with an IRed intensity of 50. The cut off time for finishing the IRed stimulation was set at 7 seconds. The latency to the paw withdrawal was recorded and 5 measurements per paw were done with an interval of 2 minutes between measurements. The individual cages and all the set up were cleaned with a 30 % ethanol solution between animals.

Von Frey test; The mechanical nociceptive response was assessed using the Von Frey paradigm (Ugo Basile, Varese, Italy). Each mouse was placed inside individual cages (10 x 10 x 14 cm) with a mesh floor where animals could freely move. During the first 20 minutes, mice were habituated to the environment. The ventral surface of left and right hind paws was stimulated with calibrated Von Frey filaments. The strength of filament application was considered enough when it buckled. If there was paw withdrawal without buckling the filament, the measurement was repeated with an interval of 30 seconds between applications. The pain threshold was determined as the tension of the filament (in grams) at which the mouse withdrew its paw in 3 out of 5 applications. The individual cages and all the set up were cleaned with a 30 % ethanol solution between animals.

Statistics

Due to the limited number of animals (n=2-4), no statistical analysis could be done. Data were expressed as mean \pm SEM.

6.3. Results

6.3.1. 6-OHDA lesion effect on weight increment and water consumption. Evaluation of ABA treatment

Since the start of ABA treatment at P28 (Fig. 6.1), weight and water intake were monitored once a week. The results are presented as the means \pm SEM (n = 2-4 per group).

In females, weight increment in sham group was from 15.70 ± 0.49 g (week 1) to 18.63 ± 0.23 g (week 4); in the 6-OHDA group from 13.90 ± 0.10 g (week 1) to 21.95 ± 1.85 g (week 4); and in 6-OHDA+ABA group from 15.12 ± 0.22 g (week 1) to 18.47 ± 0.17 g (week 4). This represents an increment of: 118.67 ± 2.40 % for sham, 158 ± 14 % for lesioned and 122.25 ± 3.09 % for lesioned + ABA (Fig. 6.2A).

In males, the sham group body weight increased from 19.70 ± 0.78 g (week 1) to 25.23 ± 0.27 g (week 4), lesioned mice from 20.77 ± 0.14 g (week 1) to 23.50 ± 0.49 g (week 4); and lesioned +ABA male exhibited the highest increment, from 15.53 ± 0.34 g (week 1) to 24.70 ± 0.51 g (week 4). This represents an increment of 128.67 ± 5.78 % for sham; 113 ± 2.08 %, for 6-OHDA, and 159 ± 6.66 % for 6-OHDA+ABA (Fig. 6.2B). These results suggest that lesion had opposite effects in males and females.

Weekly water consumption was registered per cage and it is represented by the mean (mL) of the 4 weeks (1 cage per group). In females, 6-OHDA+ABA group average water intake was 111.25 mL, followed by the sham female mice (98.75 mL) and 6-OHDA female mice (76.25 mL) (Fig. 6.2C). Males water intake was 117.5 mL for the 6-OHDA+ABA group, 97.5 mL for sham group and 93.75 mL for 6-OHDA group (Fig. 6.2D). We determined the ABA intake based on the monitoring of water consumption for the two 6-OHDA groups that received ABA: females consumption was 0.84 mg/week/cage and males 0.87 mg/week/cage (Fig. 6.2E).

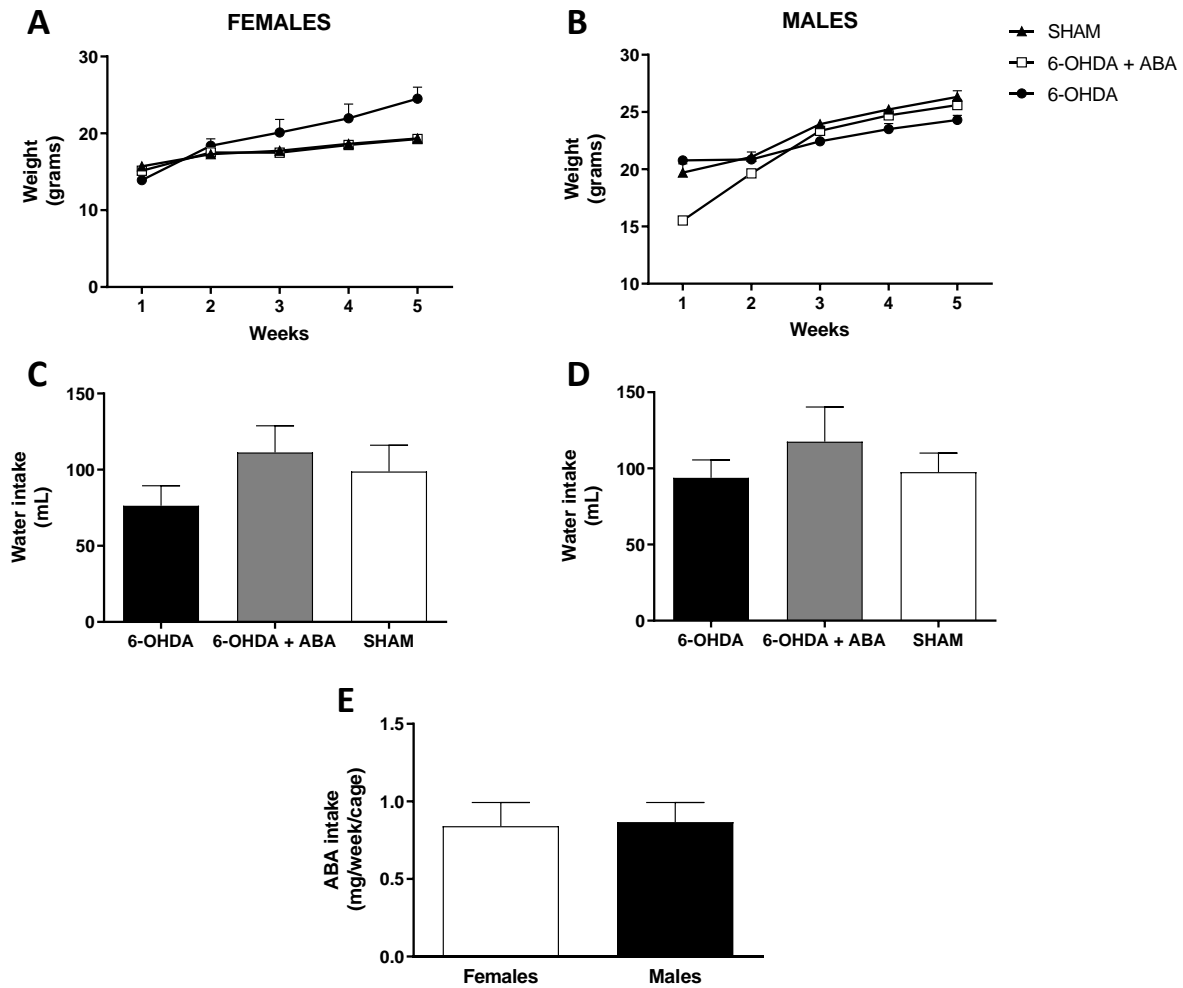


Fig. 6.2. Body weight (grams) and water intake (mL) of 6-OHDA (black circles or black columns), 6-OHDA with ABA (6-OHDA+ABA, white squares, or grey columns) and Sham (black triangles or white columns) females (A, C) and males (B, D) along the 30 days of ABA treatment (8 mg/L in the drinking water). ABA consumption of 6-OHDA-injected females and males was weekly measure per cage (E). $n = 2-4$ per group. Values are mean \pm SEM.

6.3.2. 6-OHDA lesion effect on impulsivity

Another distinctive trait of ADHD children is the impulsivity^[409,410]. The CAR test is based on the tendency of rodents to avoid falling from high surfaces, so that animals with an impulsivity disorder will be more likely to jump out from the platform. The results are presented as the means \pm SEM ($n = 3-6$ per group). In females, the sham group jumped (2.67 ± 2.67 number of falls) and 6-OHDA jumped more (6.17 ± 5.78 number of falls) (Fig. 6.3A). However, the latency to the first fall was similar in both, lesioned (46.92 ± 9.16 min) and sham (43.33 ± 16.67 min) (Fig. 6.3C). Suggesting that lesion may have increased impulsivity in females. None of the sham male mice jumped out of the platform, whereas the 6-OHDA group jumped 4.50 ± 4.11 times (Fig. 6.3B) with a latency of 47.92 ± 9.56 (Fig. 6.3D). Also in males, lesion seems to increase impulsivity.

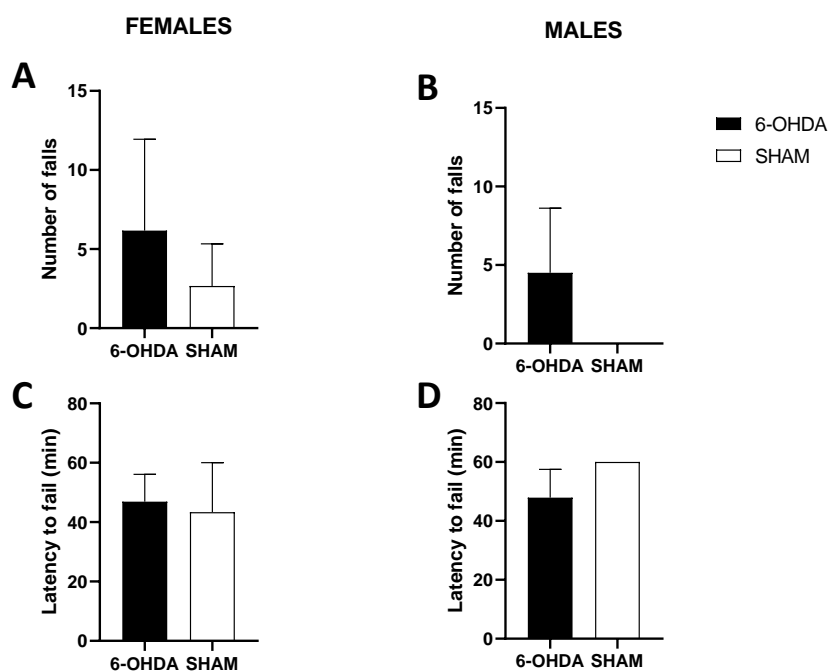


Fig. 6.3. Impulsivity. Cliff avoidance reaction (CAR) test data of 6-OHDA-injected mice (6-OHDA, black columns) ($n = 6$) and vehicle-injected mice (SHAM, white columns) ($n = 3$) for females (A, C) and males (B, D). Number of falls (A, B) and latency to the first fall (min; C, D) were recorded at P26. Data are presented as mean \pm SEM.

6.3.3. 6-OHDA lesion effect on the spontaneous locomotor hyperactivity. Evaluation of ABA treatment

One of the main and characteristic symptoms in ADHD is the spontaneous locomotor hyperactivity and it has been shown in mice^[388], rats^[411] and humans^[412]. We used the open filed paradigm to evaluate spontaneous locomotor hyperactivity in our model. Velocity (cm/sec) and distance travelled (cm) were recorded and are represented by the average of the two days of test. The results are presented as the means \pm SEM ($n = 2-4$ per group).

At P24-25, female's data for sham group were: 3.71 ± 0.31 cm/s (velocity) and 2216 ± 181.6 cm (distance); lesion decreased velocity (6-OHDA-VEH 3.26 ± 0.01 cm/s; 6-OHDA+ABA 3.41 ± 0.23 cm/s) and reduced distance (6-OHDA-VEH 1792 ± 155.2 cm; 6-OHDA+ABA 1989 ± 137.3 cm). Thus, suggesting that the neonatal lesion decreased spontaneous locomotor activity in females. Four weeks after; sham females increased velocity (4.26 ± 0.51 cm/s) and distance (2609 ± 407.10 cm) respect to P24-25. 6-OHDA-VEH females (velocity: 3.94 ± 0.73 cm/s; distance: 2316 ± 428.9 cm) also increased respect to P24-25. 6-OHDA+ABA group increased the most: velocity (5.33 ± 0.24 cm/s) and distance (3040 ± 119.5 cm) (Fig. 6.4A, B). Thus, suggesting that ABA induced hyperactivity in the lesioned group.

In males, sham activity at P24-25 had lower velocity (3.86 ± 0.14 cm/s) and distance (2307 ± 80.26 cm) than lesioned male mice velocity (6-OHDA-VEH 4.54 ± 0.44 cm/s; 6-OHDA+ABA 4.28 ± 0.63 cm/s) and distance (6-OHDA-VEH 2660 ± 262.2 cm; 6-OHDA+ABA 2552 ± 374.6 cm). Indicating that neonatal lesion had increased spontaneous locomotor activity (as expected). After 4 weeks of treatment; sham males increased activity, velocity (4.81 ± 0.39 cm/s) and distance (2780 ± 249.7 cm), but none of the lesioned groups: 6-OHDA-VEH (velocity: 4.17 ± 0.37 cm/s; distance: 2446 ± 218.9 cm) and 6-OHDA+ABA group (velocity: 4.06 ± 0.29 cm/s; distance: 2377 ± 179.30 cm) (Fig. 6.4C, D). Suggesting that ABA had no effect in the lesioned group spontaneous locomotor activity, contrary to females.

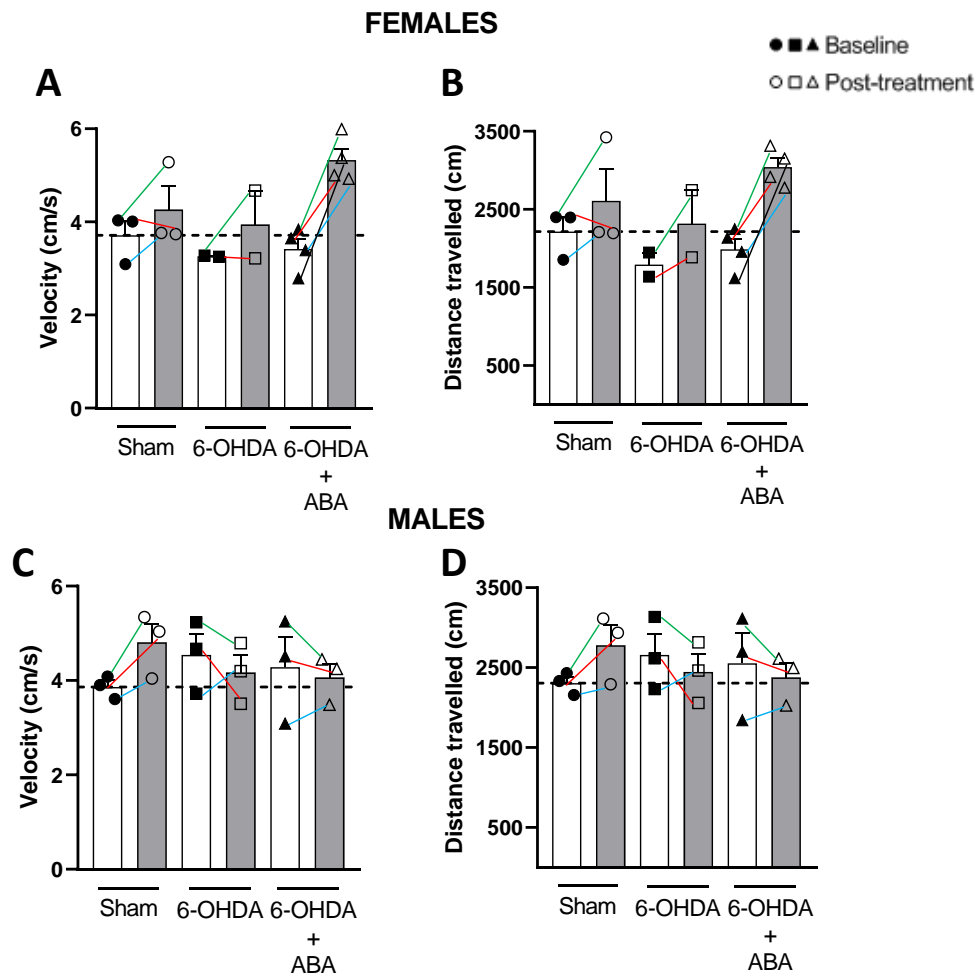


Fig. 6.4. Spontaneous locomotor hyperactivity. Open field data of 6-OHDA-injected mice (6-OHDA, squares) ($n = 2-3$), vehicle-injected mice (SHAM, circles) ($n = 3$) and 6-OHDA animals that received ABA in the drinking water (8 mg/L) (6-OHDA+ABA, triangles) ($n = 3-4$) for females (A, B) and males (C, D) at the baseline (P24-P25; white columns with filled symbols) and after 1 month of treatment (ABA or VEH) (P56-P57; grey columns with empty symbols). Velocity (cm/sec) (A, C) and distance travelled (cm) (B, D) were recorded. Values are the mean of both test days and data are presented as mean \pm SEM.

6.3.4 6-OHDA lesion effect on pain hypersensitivity. Evaluation of ABA treatment

Some clinical studies with children and adults with ADHD have reported an elevated pain perception in these patients ^[406,413,414]. To evaluate pain sensitivity, we performed two pain behaviour tests: plantar and Von Frey test. Plantar test quantifies the latency to paw withdrawal upon increasing thermal stimulus. The lower latency, the higher sensitivity to stimulus. Values are calculated by the average of 5 measures in both paws (left and right hind paws) and the results are presented as the mean \pm SEM ($n = 3-6$ per group). 6-OHDA-injection seems to lower pain threshold (as expected). Sham females stood 4.01 ± 0.10 sec without withdrew the paws and 6-OHDA females 3.68 ± 0.37 sec (Fig. 6.5A). Meanwhile, sham males had a latency score of 3.77 ± 0.93 sec and 6-OHDA males had a score of 3.17 ± 0.45 sec (Fig. 6.5B). The effect of the lesion seems to be more noticeable in males.

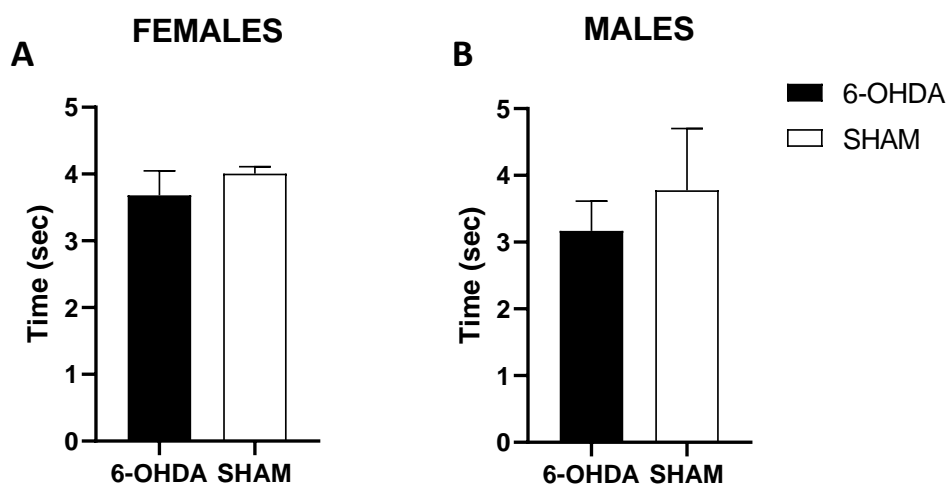


Fig. 6.5. Thermal stimulation (pain threshold). Plantar (Hargreaves apparatus) test data of 6-OHDA-injected mice (6-OHDA, black columns) ($n = 6$) and vehicle-injected mice (SHAM, white columns) ($n = 3$) for females (A) and males (B). Latency (sec) to the paw withdrawal under a thermal stimulus (IRed intensity of 50) was recorded at P27 (baseline). Values are the mean of 5 measurements and both paws (left hind paw and right hind paw). Data are presented as mean \pm SEM.

Unlike the plantar test, Von Frey test uses a mechanical stimulus to induce pain. The tension of the filament (in grams) at which the animal withdrew the paw was recorded and results are presented as the average of the two hind paws. Values are expressed as the means \pm SEM ($n = 2-4$ per group). To evaluate the effect of ABA treatment animals were subjected to the Von Frey test at P27 (baseline) and P58 (post-treatment).

Sham females could not be correctly measured at P28, but at P58, showed a pain threshold of 1.23 ± 0.23 g. After a 4-week treatment of VEH or ABA, the 6-OHDA+ABA female group lower the threshold (0.95 ± 0.09 g) compared to 6-OHDA-VEH (1.35 ± 0.35 g), suggesting a potential increased of pain sensitization by ABA treatment (Fig. 6.6A).

In males, sham displayed a pain threshold of 2.07 ± 0.98 g at P28 and after 4 weeks threshold was lower, 1.47 ± 0.14 g (Fig. 6.6B). The 6-OHDA males showed a baseline pain threshold of 1.40 ± 0.10 g (6-OHDA in Fig. 6.6) and 1.13 ± 0.13 g (6-OHDA+ABA in Fig. 6.6) and, after 4 weeks, it was much lower (6-OHDA 0.87 ± 0.07 g), whereas 6-OHDA+ABA males were equal to P58 (1.23 ± 0.12 g); suggesting that ABA may counteract lesion effect in pain. Opposite to what we observed in females.

This pilot study indicates that perhaps ABA treatment may have totally opposite effects in both sexes. The confirmation of these preliminary results is of huge relevance in the understanding of sex differences underlying disease symptoms, and to design effective treatments.

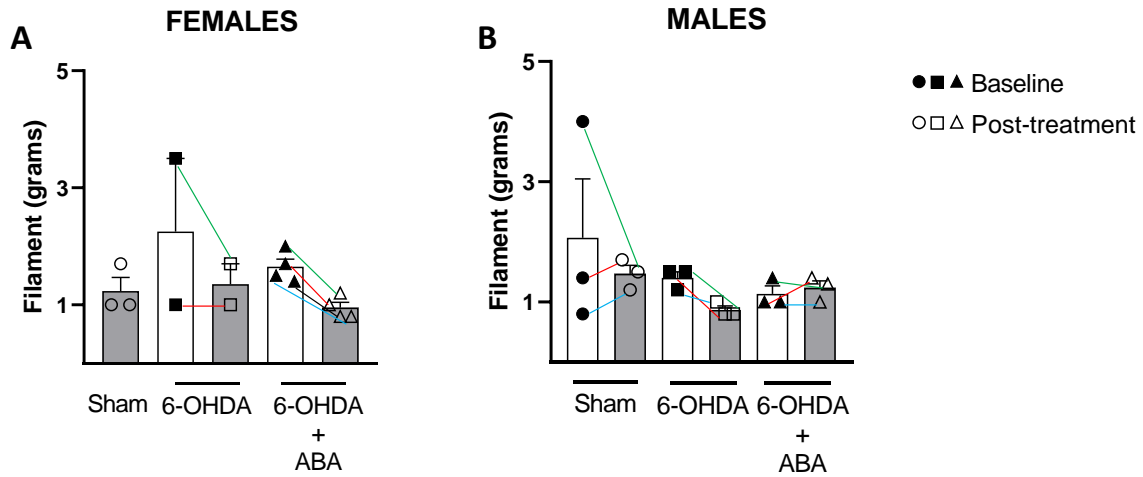


Fig. 6.6. Mechanical stimulation (pain threshold). Von Frey test data of 6-OHDA-injected mice (6-OHDA, squares) ($n = 2-3$), vehicle-injected mice (SHAM, circles) ($n = 3$) and 6-OHDA animals that received ABA in the drinking water (8 mg/L) (6-OHDA+ABA, triangles) ($n = 3-4$) for females (A) and males (B) at the baseline (P28; white columns with filled symbols) and after 1 month of treatment (ABA or VEH) (P58; grey columns with empty symbols). The pain threshold based on calibrated filament tension (in grams) was recorded. Values are the mean of both paws (left hind paw and right hind paw). Data are presented as mean \pm SEM.

6.4. Discussion

In this pilot study, we evaluated the effect of ABA, a phytohormone with anti-inflammatory properties, in a 6-OHDA neonatal lesion model of ADHD in females and male mice. The aim is to ascertain if ABA can ameliorate the symptoms in psychiatric disease conditions, as we have shown for learning and memory tasks in animal models of inflammatory conditions.

6-OHDA lesion model is a validated Parkinson's disease (PD) model due to its specific neurotoxicity against dopaminergic neurons^[415,416]. This DA depletion in neonatal mice has been demonstrated to induce ADHD-like behaviour^[388]. To assess if we could counteract ADHD symptoms through reducing neuroinflammation, we treated animals with ABA in the drinking water (8 mg/L). All groups showed a similar water intake (as expected by our previous studies). Weight was also monitored. Males had a higher body weight gain compared with females, as expected for rodents. Interestingly, 6-OHDA-injected females showed bigger weight increment than sham, reaching male weight by two months. However, lesioned males had lower weight increment. The effect on females has never been reported before, thus pointing to a putative effect of sex in the association between dopaminergic system alterations, and body weight gain and obesity^[417,418]. Some studies indicate that hormones such as estradiol promote oxidative stress and DA depletion in females Hyp, which is an area involved in feeding behaviour^[419]. Thus, females could be more sensitive to DA reduction effects on weight. Males with lesion, on the other hand had lower weight than sham, perhaps due to increased activity.

Most interestingly, ABA administration seems to counteract the effect on body weight in both lesioned groups, that is decreasing increment in the females and increasing it in males.

To evaluate ADHD symptoms, we measured the performance of all mice in different paradigms: hyperactivity (open field paradigm), impulsivity (CAR test), and pain sensitivity (plantar and Von Frey tests).

In the open field paradigm, the more distance travelled, and the higher speed indicates higher spontaneous locomotor activity. We consider that sham animals with no treatment is the "normal behaviour" for this strain in these conditions. We have observed that lesion seemed to increase speed and distance in males, as reported^[388,420], however it seems to reduce activity in females. If we confirm this preliminary data, this finding had never been reported before and constitutes a considerable sex difference in the response to DA lesion.

In addition, both female and male controls increased activity after 4 weeks (no treatment), while ABA treatment does not seem to affect locomotor activity in lesioned males, it increases in females. This could suggest sex-dependent effects of ABA in lesioned subjects. Some studies have reported sex-differences in nutraceuticals' action (e.g. curcumin, also a PPAR γ activator^[421]), Perhaps due to different metabolism absorption^[422]. Thus, we cannot rule out that possibility.

In the CAR test, impulsivity is measured by the jumps: the more jumps and lower latency to jump, the higher impulsivity. Control females had higher impulsivity compared to control males. Lesion had no effect in females, but increased impulsivity in males (as expected). To our knowledge this sex differences in impulsivity had never been reported before. The test was not performed after VEH or ABA treatment, so we cannot conclude the ABA effect on it.

In our pilot study, we have shown that the dopaminergic lesion model may affect differently males and females hyperactivity and impulsivity. Some studies indicates that there are sex differences in the striatum DA synthesis, so the impact of DA depletion can differ in males and females brain circuitry^[423]. Of course, the confirmation of DA lesion must be done as well, to ascertain the reach of lesion and areas in both sexes.

Inflammation and pain are two closely linked fields. In this pilot study, plantar test results suggested that sham females have higher threshold than sham males (more tolerant to pain). Males inoculated with 6-OHDA had increased pain sensitization compared with sham controls (as

expected), but this effect seemed smaller in females. Von Frey test was done at P27 and P58; we observed that ABA prevented the lesion effect in males (thus ameliorating pain sensitization) but not in females, where it had the opposite effect. If these data were to be confirmed, it would mean that ABA, perhaps increasing PPAR γ , could be associated to higher pain perception in females. Similarly, increased PPAR γ has been found associated to pain in women suffering peritoneal endometriotic lesions^[424]. Thus, we could speculate that the excessive PPAR γ -agonist action might exert negative effects in females and therefore ABA treatment for pain must be carefully evaluated.

Up until now, there are no reports about C57BL/6 mice strain in neonatal 6-OHDA-lesioned model. Another major novelty of this pilot study is the comparative analysis of this model in female and male mice. Moreover, these preliminary results point to sex differences in ADHD symptomatology, pain perception and, importantly, in therapeutic strategy.

7

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Our findings confirm the relevance of insulin resistance and inflammation inducing memory alterations, and how ABA, a molecule with insulin-sensitizing and anti-inflammatory properties is a potential treatment. Moreover, we dissect insulin resistance from inflammation, and proved that reduced IRS1 expression impairs spatial memory, in the absence of inflammation. In that study, we started to point to important sex-differences in susceptibility to IRS1 deficits. Finally, in our pilot study, we further confirm the absolute necessity to carry out comparative studies in both females and males, with particular emphasis on therapeutic interventions.

7.1. The potential therapeutic value of ABA, in male rodents

In this thesis, we confirm that ABA administered in the drinking water improved peripheral glucose tolerance and cognitive alterations in a model of inflammation induced by HFD. T-maze and novel object recognition paradigms have been validated as reliable spatial and recognition memory tasks respectively ^[210,234]. We would need to analyse whether the progression of inflammation affects sequentially different types of memory functions, as happens in AD patients with an early impaired episodic memory ^[425], then ABA rescue effect is limited in time depending on disease progression.

Impaired IRS1 or IRS2 response can lead to insulin resistance. In our work, we demonstrated for the first time that HFD differently alters hippocampal IRS1 and IRS2 mRNA expression, IRS2 expression is reduced whereas IRS1 expression is increased, which could indicate different roles in neuroinflammation and insulin resistance. We show that ABA counteracts this effect in addition to reduce HFD-induced inflammatory markers (reactive microglia and pro-inflammatory cytokines), improved hippocampal neurogenesis, and restored APP (increased by HFD) and BDNF (reduced by HFD) expression. We concluded that ABA treatment may have an effect on the mRNA promoter activity of these genes, likely via PPAR γ activation ^[147], in accordance with previous data, showing that PPAR γ agonists administration in APP/PS1 and diabetic transgenic mice restored IRS1 ^[299] and IRS2 ^[144] expression, respectively.

Other PPAR γ agonist (synthetic insulin sensitizer molecules, e.g. TZDs) have been reported to reduce pro-inflammatory cytokines in patients with rheumatoid arthritis ^[426]. However, these drugs have undesirable side effects ^[195-197]. ABA shares molecular mechanisms with TZDs, but we postulate that ABA treatment could be a safer natural alternative to synthetic insulin sensitizer molecules.

Finally, we consider that the effects on hippocampal BDNF, IRS1, IRS2, APP, cytokines expression and neurogenesis are critical for the cognitive beneficial properties of ABA, however, whether is a peripheral benefit effect on brain or a direct effect on brain neuronal and astroglia cells is currently unknown. In addition, we would like to discern if ABA action as insulin sensitizer downregulates inflammation or is the anti-inflammatory properties that lower inflammation.

Moreover, the effect of ABA in control animals, sometimes worsen the results, is very interesting and points to optimal levels not lower, but also not higher, if we consider that ABA can be synthesized endogenously in healthy individuals, then we can infer that we are “adding” ABA perhaps too higher than optimal levels. The optimal range need to be determined.

Last, but not least, the effect of ABA on female rats, both controls and fed with a HFD need to be elucidated.

7.2. Insulin resistance impairs spatial and recognition memory in females, only spatial memories in males

Insulin resistance animal models and clinical diseases are usually accompanied by increased inflammation, thus the relative contribution to cognitive impairment is uncertain. In this thesis, we have elucidated the effect of reducing IRS1 signalling on cognitive function, in the absence of inflammation, by using an adeno-associated virus (AAV) expressing shRNA targeting IRS1 mRNA to knock down its expression specifically. Whether this IRS1 reduction resulted in “insulin resistance” was only inferred by the western blot analysis in cultured cells treated with insulin, but it would need to be determined *in vivo*. Moreover, whether this IRS1 reduction increases inflammatory markers or worsen a possible inflammatory effect of stereotaxic injection, also remains to be elucidated.

There is an association of hippocampal alterations with clinical anxiety and depression [427,428]. In rodents, this function is associated with ventral HPC [429], area not affected by our AAV-injection (data not shown), which may explain why we did not find these symptoms in our model. We observed that AAV DJ8 virus affects mostly GABAergic hippocampal neurons which inhibit locally and to other areas, such as the MS. Since this area is particularly involved in memory function, we consider that specific intervention on this circuit may contribute to the absence of anxiety or depressive symptoms. AAV inoculations affecting ventral HPC would be required to answer that question. Moreover, the AAV shIRS1 will help us to analyse the effects of IRS1 knocking down in other brain areas. Furthermore, the comparative study of IRS2 in same circuit remains to be elucidated.

We have revised that insulin signalling promotes neuronal survival and energy homeostasis [31,110–112,430,431], and it is required for neuroplasticity and spatial memory [32]. Insulin is also required for glutamate receptors [25,27] and PSD-95 expression at the surface membrane [29], and optimal synaptic plasticity [432]. Our data indicates that the roles reported for IR in synaptic plasticity could be mediated by IRS1, since we have found that IRS1 downregulation reduces synaptic plasticity by indirect measures of presynaptic marker (synaptophysin). The molecular mechanism underlying synaptophysin alteration by IRS1 silencing is unknown. Since IRS1 is required for PI3K-Akt pathway-induced GLUT4 translocation to plasma membrane [433], we postulate that this pathway is involved in preventing the extra glucose needed for activity, perhaps due to the translocation of other synaptic proteins. These mechanisms remain to be elucidated.

Furthermore, IRS1 downregulation could also induce postsynaptic activity alterations, thus it should be analysed in further studies for instance through electrophysiological techniques that would help to better understand the effects on both pre and postsynaptic currents.

In our work, we used AAV DJ8 serotype, which has shown a preference for GABAergic neurons. Thus, the effect of IRS1 silencing in glutamatergic neurons remains to be elucidated. This can be achieved by, for example, changing AAV tropism (different serotypes) to improve infection in different neuronal types.

Comparative studies of females and males are still minority in preclinical field. Still, different meta-analyses unveiled that males performs better spatial navigation and working memory tasks than females, in clinical [434] (pointing to social as well as biological differences), but also in preclinical studies [435], (suggesting just biological differences). In patients suffering dementia, women displayed worse memory deficits [436] and faster temporal lobe atrophy [437] than men, and the prevalence of AD is higher in women [346]. Furthermore, insulin resistance can be induced by hormonal alterations (e.g. polycystic ovary syndrome) in absence of obesity condition [438]. Also estrogen levels are associated with less-effective transport of insulin into the CNS [439].

In this thesis, we carry out an approach to evaluate sex differences in memory tasks. We found that IRS1 reduction affected spatial memory in both females and males, whereas recognition memory was only affected in females. Our study was conducted with non-ovariectomized rats, thus the insulin resistance susceptibility difference may be due to hormonal modulated events. These

results confirm that insulin resistance may have a higher impact in females. Whether this is due to estrogen synergy with IRS1 deficits (e.g. mitochondria function ^[440], AMPA receptors stoichiometry ^[441], spine density of pyramidal neurons ^[442], LTP and or LTD ^[443]) remains unknown and it is an objective in future research.

7.3. ADHD model: potential sex-differences

Even with different aetiology than neurological diseases, psychiatric disorders have also been associated to neuroinflammation, e.g. major depressive disorder (MDD) ^[444], bipolar disorder ^[445]. In this thesis, we show the preliminary data of a pilot study of ABA effects on a model of ADHD in both males and females. To our knowledge, it is the first comparative study attempting to associate inflammation and ADHD in preclinical model, and pain sensitization in both female and males.

Hyperexcitability derived of neuroinflammation in spinal cord and brain causes pain central sensitization ^[446]. Specifically, pro-inflammatory cytokines promote hypersensitivity of nociceptors (pain receptors) ^[447]. The continuous releasing of those inflammatory cytokines leads to chronic widespread pain that is extended to mostly all the body ^[447] and can result in chronic pain disease ^[448]. Our data suggests that this model may be a useful tool to understand ADHD and pain relationship.

Early exposure to inflammatory processes may be a possible risk factor to develop ADHD ^[390,449-451]. Also, growing evidence suggests that there is a link between pain and ADHD ^[452-455], but it is still a relatively unexplored field, especially in preclinical studies. Thus, we hypothesized that anti-inflammatory treatments could be at the crossroad of ADHD traits and pain symptoms, then ameliorating the disease. However, our data supports that same treatment may not be used equally in both females and males.

In other preclinical studies, estradiol can promote the inflammatory response by increasing GFAP and IL-1 β expression; oxidative stress and DA depletion in Hyp ^[419]. Also in humans, sex differences have been found, supporting that ADHD prevalence is higher in males than females ^[456,457] also with different symptomatology: whereas impulsivity is prevalent in males, inattention trait seems predominant in women ^[458]. However, in humans there are not only biological differences, but also social and familiar influences.

Our data support sex-differences in basal behavior, lesion-induced behavior and even treatment effects. Thus, tentatively we suggest that: i) females are more active and impulsive than male in the basal state; ii) neonatal DA depletion may not promote hyperactivity/impulsivity traits in females, as it does in males, iii) ABA treatment seems to improve ADHD behavioural traits in males but not females, where it seems to even worsen them, iv) ABA appears to counteract the body weight gain observed in females, perhaps by increasing hyperactivity. Therefore, further studies should be carried out to understand the underlying mechanisms. This would be necessary for development of suitable anti-inflammatory treatments against ADHD symptoms.

If these results are confirmed, they will greatly contribute to further understand sex differences in the neuroinflammatory etiology of ADHD and whether ABA could be a potential treatment to ameliorate classic traits and pain hypersensitization. Even though sex differences in inflammatory response are known (e.g. differences in circulating monocytes ^[459] and microglia ^[460,461]), the mechanisms explaining potential opposite effects of ABA remains to be elucidated.

These data strongly endorse to always carry out comparative studies with females and males, since treatments and symptomatology may have very different outcomes under certain conditions.

8

CONCLUSIONS

ABA treatment in a male rat model of high fat diet-induced neuroinflammation:

- 1) restores spatial and recognition memory function, concomitant with increasing peripheral insulin sensitivity.
- 2) crosses the blood brain barrier.
- 3) reduces neuroinflammation in hypothalamus and hippocampus.
- 4) improves neurogenesis in hippocampus.
- 5) restores hippocampal IRS1 and IRS2 expression levels that are differentially affected in this model.
- 6) restores hippocampal BDNF and APP expression levels in hippocampus, affected by this model.
- 7) in sham animals may worsen some parameters, pointing to a window of beneficial action.

AAV-CMV-shIRS1-EGFP inoculation in dorsal hippocampus, compared to AAV-CMV-EGFP control:

- 8) both labelled preferentially hilar GABAergic neurons.
- 9) reduces IRS1 expression in hippocampal hilar neurons.
- 10) in females is sufficient to cause spatial and recognition memory deficits in the absence of neuroinflammation.
- 11) in males causes only spatial memory alteration in the absence of neuroinflammation.
- 12) in females modulates the synaptic plasticity in GABAergic hippocampal neurons projecting to medial septum.
- 13) does not alter anxiety symptomatology.

ABA treatment in a 6-OHDA neonatal lesion model of ADHD (Pilot study):

- 14) highlights the potential differences due to sex-differences in drug effects and/or brain lesions models, and the need to evaluate these in both sexes. Points to a revision of disease preclinical models and pharmacological treatments in comparative females vs males studies.

9

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10

ANNEXES

9.1. Supplementary information

9.1.1. Cell transfection and RT-qPCR

HEK cells were grown at 37°C in a 5% CO₂ incubator and 90% humidity and were free of mycoplasma contamination. Cells were transfected by the calcium chloride method with plasmids control pSM155-CMV-EGFP generous donation of Dr Melanie White and our generated pSM155-CMV-shIRS1-EGFP with two different sequences (shIRS1A, shIRS1B). To quantify the knockdown capacity of the shRNA candidates, we transfected the HEK293 with the shRNA containing plasmids using the calcium phosphate method and measure the IRS1 and IRS2 expression using real-time PCR. GAPDH was used as an internal control. The sequences of the primers used are depicted in Supplementary Table S5.2.

9.2. Supplementary figures

sh-IRS1A

Top

5'TGCTGGCAGGCACCATCTCAACAATCGTTTTGGCCACTGACTGACGATTGTTGAGA
TGGTGCCTGC 3'

Bottom

5'CCTGGCAGGCACCATCTCAACAATCGTCAGTCAGTGGCCAAAACGATTGTTGAGAT
GGTGCCTGCC 3'

sh-IRS1B

Top

5'TGCTGGCCTGGAGTATTATGAGAACGGTTTTGGCCACTGACTGACCGTTCTCATAA
TACTCCAGGC 3'

Bottom

5'CCTGGCCTGGAGTATTATGAGAACGGTCAGTCAGTGGCCAAAACCGTTCTCATAAT
ACTCCAGGCC3'

Fig. S5.1. Sequences of the oligonucleotides used for the two shRNA constructs selected as shIRS1 candidates.

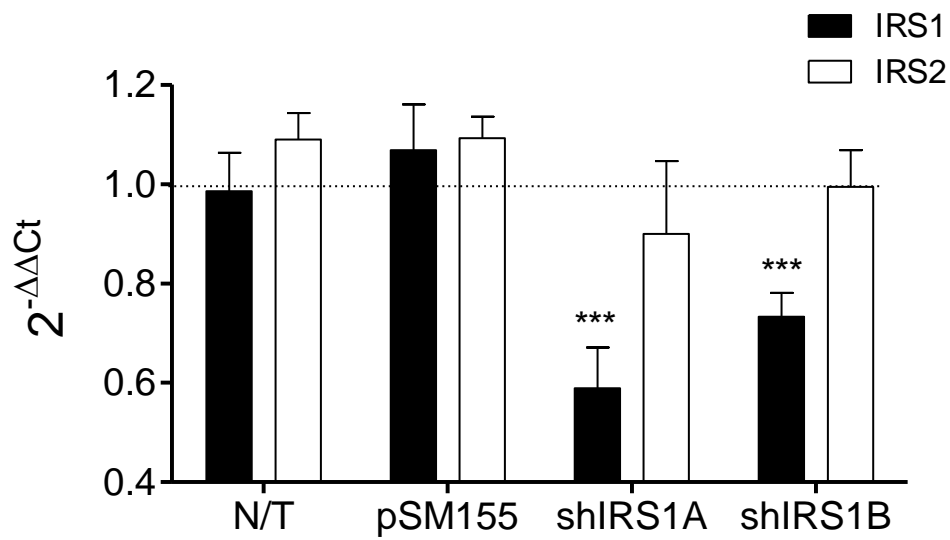


Fig. S5.2. Silencing effect of shIRS1 in cultured cells. Human embryonic kidney cells (HEK 293) were transfected with the different plasmids as indicated. IRS1 and IRS2 mRNA expression was quantified by qPCR. Data were analyzed using two tailed Mann Whitney t-test; *** $p < 0.001$ compared to control plasmid pSM155.

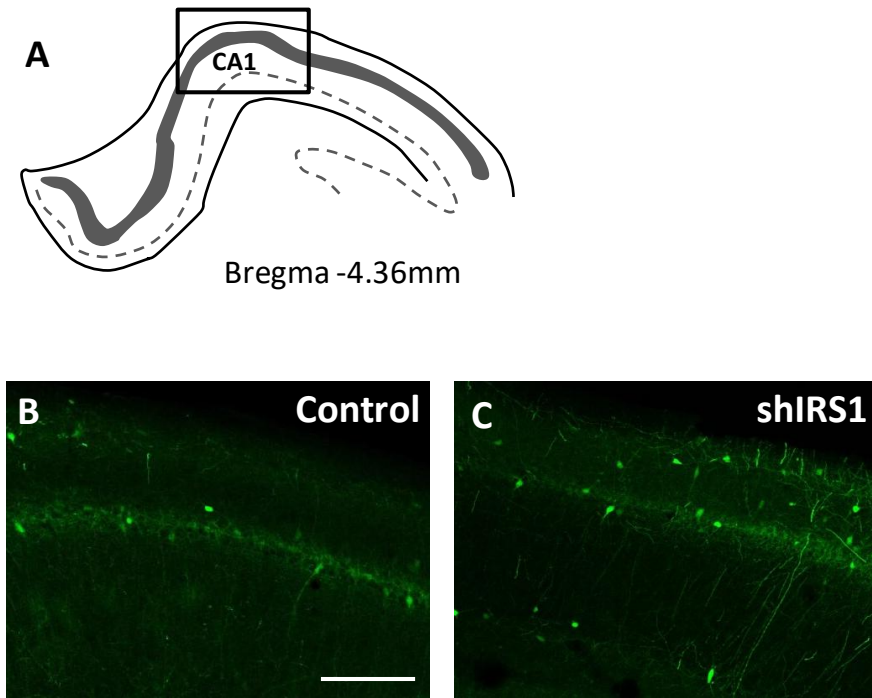


Fig. S5.3. AAV in the hippocampus CA1 field. Schematic representation of CA1 of the dorsal hippocampus (A). Scattered control (B) and shIRS1 (C) labeled neurons were observed in the hippocampus CA1. Scale bar=100 μ m (n=3-5).

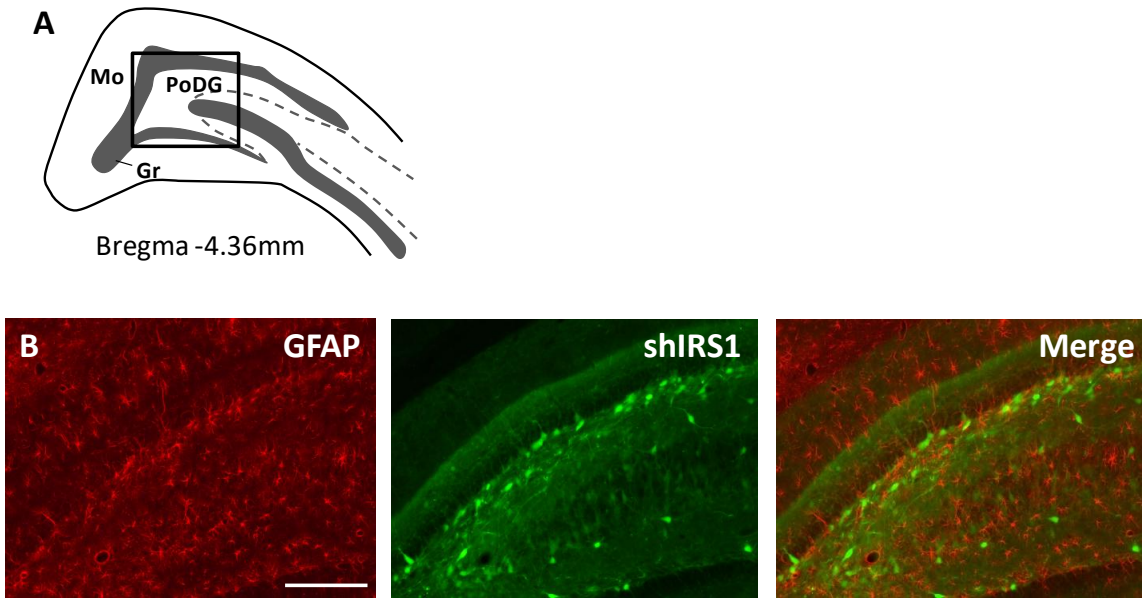
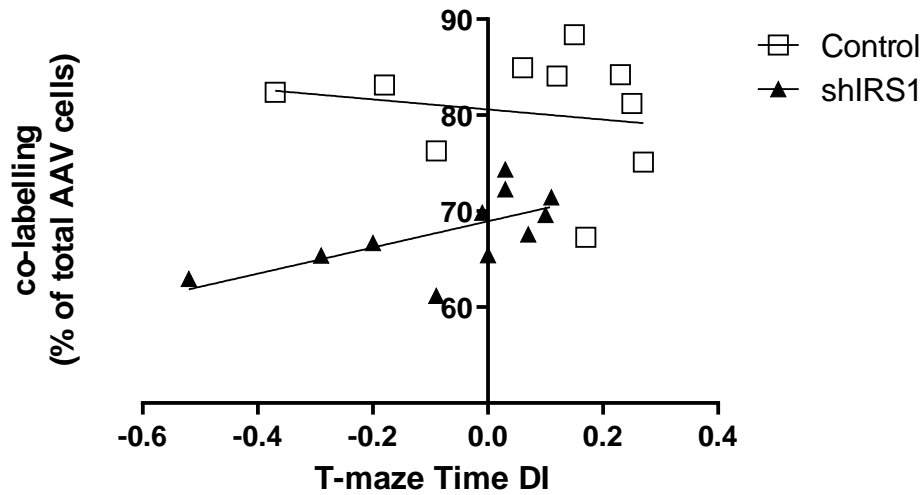


Fig. S5.4. AAV DJ8 does not colocalize with GFAP-positive astrocytes. Schematic representation of the dentate gyrus of the dorsal hippocampus (A). We found no co-localization of AAV labeled cells with Glial Fibrillary Acidic Protein (GFAP) positive cells (B). Scale bar=100 μm (n=3-4).



	Time DI vs Control	Time DI vs shIRS1
Pearson r (p value)	-0.15 ($p = 0,34$)	0.63 ($p = 0,018$)
Slope significant from zero	No	Yes

Fig. S5.5. Correlation between T-maze behaviour (DI) and IRS1 knockdown was compared between control subjects (squares) and shIRS1 (triangles). Pearson r values and probability are indicated, only in shIRS1-inoculated animals, behaviour correlated positively with IRS1 expression correlates p Regression analysis indicated that the slope is significantly different from zero only in shIRS1-inoculated subjects.

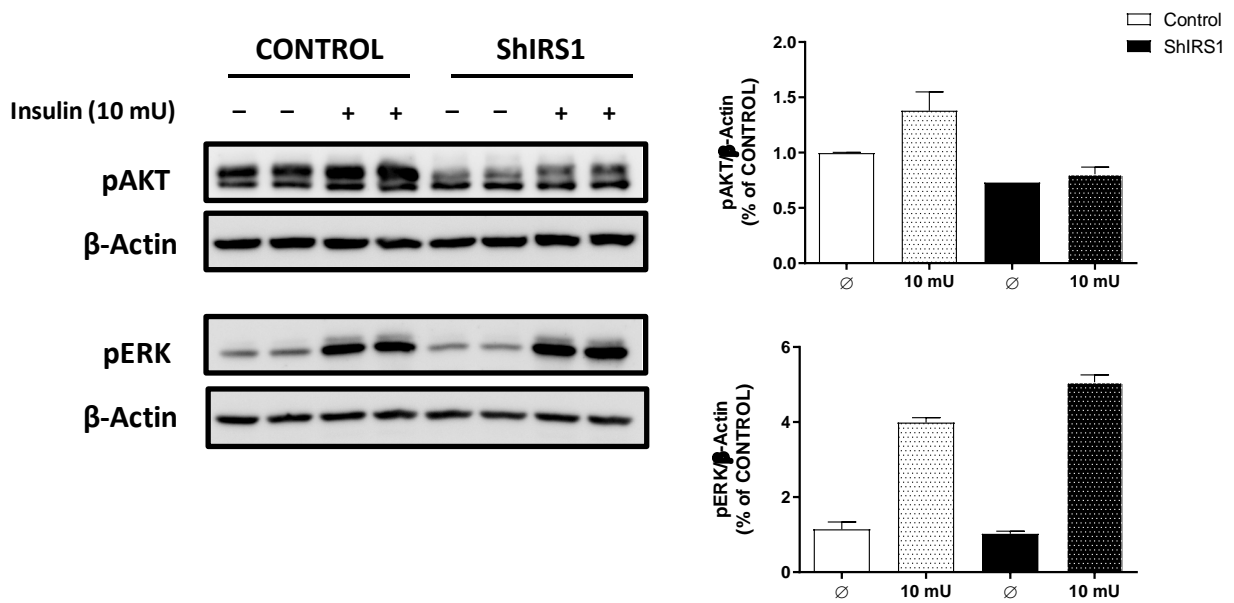


Fig. S5.6. HEK cells were transfected with shIRS1 plasmid or control plasmid. Insulin was added for 5 min and cells were collected. The AAV shIRS1 reduced levels of phosphorylated AKT (pAKT) basal and activation. pAKT: phosphorylated AKT; pERK: phosphorylated extracellular signal-regulated kinase. *Unpublished results.*

9.3. Supplementary tables

Table S5.1. Animals were weighed before surgery (Day 0) and one month after (Day 33), just prior to behavior. Subjects, females and males were classified randomly into control AAV-CMV-EGFP and AAV-CMV-shIRS1-EGFP experimental groups.

Rat	Sex	Experimental group	Weight prior to surgery (g)	Weight 33 days after surgery (prior to behavior) (g)
1	F	Control	331	332
2	F	Control	288	303
3	F	Control	308	316
4	F	Control	299	313
5	F	Control	329	330
6	F	Control	285	310
7	F	Control	293	293
8	F	ShIRS1	289	286
9	F	ShIRS1	315	315
10	F	ShIRS1	298	299
11	F	ShIRS1	263	279
12	F	ShIRS1	335	344
13	F	ShIRS1	344	340
14	F	ShIRS1	295	293
15	F	ShIRS1	306	312
1	M	Control	460	503
2	M	Control	485	565
3	M	Control	523	572
4	M	Control	455	540
5	M	Control	495	536
6	M	Control	463	505
7	M	Control	450	527
8	M	Control	477	542
9	M	ShIRS1	482	539
10	M	ShIRS1	478	566
11	M	ShIRS1	535	600
12	M	ShIRS1	463	511
13	M	ShIRS1	494	569
14	M	ShIRS1	517	577
15	M	ShIRS1	503	556

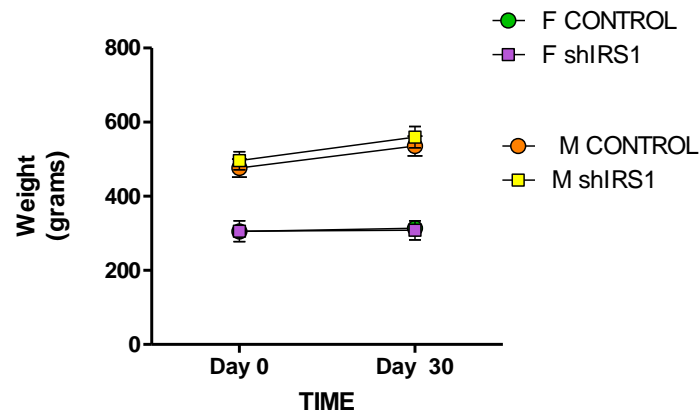


Table S5.2. Primers sequences obtained using Primer3 software tool.

Gene	Accession number	Forward Primer (5'->3')	Reverse Primer (5'->3')
GAPDH	NM_017008.4	TGCCCCCATGTTTGTGATG	TGGTGGTGCAGGATGCATT
IRS-1	NM_012969.1	ATTGGAGGTGGGTCTTGCA	TGGGGATCTTCTGGGCCATA
IRS-2	NM_001168633.1	TCACCACAGGACACAGATGC	GCATGAAGTGTGGCAAACGT

9.4. Publications

Sánchez-Sarasúa *et al. Nutrition & Metabolism* (2016) 13:73
DOI 10.1186/s12986-016-0137-3

Nutrition & Metabolism

RESEARCH

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The effect of abscisic acid chronic treatment on neuroinflammatory markers and memory in a rat model of high-fat diet induced neuroinflammation

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Abstract

Background: Western diet and lifestyle are associated with overweight, obesity, and type 2 diabetes, which, in turn, are correlated with neuroinflammation processes. Exercise and a healthy diet are important in the prevention of these disorders. However, molecules inhibiting neuroinflammation might also be efficacious in the prevention and/or treatment of neurological disorders of inflammatory etiology. The abscisic acid (ABA) is a phytohormone involved in hydric-stress responses. This compound is not only found in plants but also in other organisms, including mammals. In rodents, ABA can play a beneficial role in the regulation of peripheral immune response and insulin action. Thus, we hypothesized that chronic ABA administration might exert a protective effect in a model of neuroinflammation induced by high-fat diet (HFD).

Methods: Male Wistar rats were fed with standard diet or HFD with or without ABA in the drinking water for 12 weeks. Glucose tolerance test and behavioral paradigms were performed to evaluate the peripheral and central effects of treatments. One-Way ANOVA was performed analyzed statistical differences between groups.

Results: The HFD induced insulin resistance peripherally and increased the levels of proinflammatory markers in the brain. We observed that ABA restored glucose tolerance in HFD-fed rats, as expected. In addition, chronic ABA treatment rescued cognitive performance in these animals, while not affecting control diet fed animals. Moreover, it counteracted the changes induced by HFD in the hypothalamus; microglia activations and TNF α mRNA levels.

Conclusion: These results suggest that ABA might become a new therapeutic molecule improving the neuroinflammatory status and insulin resistance.

Keywords: Obesity, Working memory, Microglia, Phytohormones

Background

Obesity, a leading cause of type 2 diabetes [1], correlates with cognitive impairment. Insulin sensitizers have been proposed as a promising tool for the reduction of obesity-induced insulin resistance and inflammation processes. The thiazolidinediones (TZD) are a family of

synthetic insulin sensitizer molecules; however, some of them have undesirable side effects [2–4]. Thus, alternative compounds with analogous properties but fewer side effects are needed. The phytohormone abscisic acid (ABA) was found in mammalian cells more than 25 years ago [5]. Since then, several studies have proposed it as a universal signaling molecule [6, 7]. Structurally, ABA is very similar to the TZDs. Indeed, ABA can improve glucose tolerance [8], reduce the level of TNF α , and decrease adipocyte cell size in an *in vivo* model of obesity induced by HFD [9]. Moreover, in human and murine

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Abscisic Acid Supplementation Rescues High Fat Diet-Induced Alterations in Hippocampal Inflammation and IRSs Expression

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Abstract

Accumulated evidence indicates that neuroinflammation induces insulin resistance in the brain. Moreover, both processes are intimately linked to neurodegenerative disorders, including Alzheimer's disease. Potential mechanisms underlying insulin resistance include serine phosphorylation of the insulin receptor substrate (IRS) or insulin receptor (IR) misallocation. However, only a few studies have focused on IRS expression in the brain and its modulation in neuroinflammatory processes. This study used the high-fat diet (HFD) model of neuroinflammation to study the alterations of IR, an insulin-like growth factor receptor (IGF1R) and IRS expressions in the hippocampus. We observed that HFD effectively reduced mRNA and protein IRS2 expression. In contrast, a HFD induced the upregulation of the IRS1 mRNA levels, but did not alter an IR and IGF1R expression. As expected, we observed that a HFD increased hippocampal tumor necrosis factor alpha (TNF α) and amyloid precursor protein (APP) levels while reducing brain-derived neurotrophic factor (BDNF) expression and neurogenesis. Interestingly, we found that TNF α correlated positively with IRS1 and negatively with IRS2, whereas APP levels correlated positively only with IRS1 but not IRS2. These results indicate that IRS1 and IRS2 hippocampal expression can be affected differently by HFD-induced neuroinflammation. In addition, we aimed to establish whether abscisic acid (ABA) can rescue hippocampal IRS1 and IRS2 expression, as we had previously shown that ABA supplementation prevents memory impairments and improves neuroinflammation induced by a HFD. In this study, ABA restored HFD-induced hippocampal alterations, including IRS1 and IRS2 expression, TNF α , APP, and BDNF levels and neurogenesis. In conclusion, this study highlights different regulations of hippocampal IRS1 and IRS2 expression using a HFD, indicating the important differences of these scaffolding proteins, and strongly supports ABA therapeutic effects.

Keywords Insulin resistance · Hippocampus · APP · BDNF · Neurogenesis · Neuroinflammation

Abbreviations

ABA	Abscisic acid	PFA	Paraformaldehyde
ANOVA	Analysis of variance	PPAR- γ	Peroxisome proliferator-activated receptor gamma
APP	Amyloid precursor protein	RTqPCR	Real-time quantitative polymerase chain reaction
BDNF	Brain-derived neurotrophic factor	SEM	Standard error of mean
ERK	Extracellular regulated kinases	SD	Standard diet
HFD	High-fat diet	SGZ	Subgranular zone
IGF1	Insulin-like growth factor	TBS	Tris-buffered saline
IGF1R	Insulin-like growth factor receptor	TDZ	Thiazolidinediones
IR	Insulin receptor	TNF α	Tumor necrosis factor alpha
IRS	Insulin receptor substrate		

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Introduction

Adult neurogenesis is a sensitive process that is very susceptible to different toxic insults, such as the presence of beta



AAV delivery of shRNA against IRS1 in GABAergic neurons in rat hippocampus impairs spatial memory in females and male rats

Sandra Sánchez-Sarasúa¹ · Alberto Ribes-Navarro^{1,2} · María Teresa Beltrán-Bretones¹ · Ana María Sánchez-Pérez¹ Received: 4 March 2020 / Accepted: 6 October 2020
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Abstract

Brain insulin resistance is a major factor leading to impaired cognitive function and it is considered as the onset of Alzheimer's disease. Insulin resistance is intimately linked to inflammatory conditions, many studies have revealed how pro-inflammatory cytokines lead to insulin resistance, by inhibiting IRS1 function. Thus, the dysfunction of insulin signaling is concomitant with inflammatory biomarkers. However, the specific effect of IRS1 impaired function in otherwise healthy brain has not been dissected out. So, we decided in our study, to study the specific role of IRS1 in the hippocampus, in the absence of comorbidities. To that end, shRNA against rat and human IRS1 was designed and tested in cultured HEK cells to evaluate mRNA levels and specificity. The best candidate sequence was encapsulated in an AAV vector (strain DJ8) under the control of the cytomegalovirus promoter and together with the green fluorescent protein gene as a reporter. AAV-CMV-shIRS1-EGFP and control AAV-CMV-EGFP were inoculated into the dorsal hippocampus of female and male Wistar rats. One month later, animals undertook a battery of behavioral paradigms evaluating spatial and social memory and anxiety. Our results suggest that females displayed increased susceptibility to AAV-shIRS1 in the novel recognition object paradigm; whereas both females and males show impaired performance in the T maze when infected with AAV-shIRS1 compared to control. Anxiety parameters were not affected by AAV-shIRS1 infection. We observed specific fluorescence within the hilum of the dentate gyrus, in immuno-characterized parvalbumin and somatostatin neurons. AAV DJ8 did not enter astrocytes. Intense green fibers were found in the fornix, mammillary bodies, and in the medial septum indicating that hippocampal efferent had been efficiently targeted by the AAV DJ8 infection. We observed that AAV-shIRS1 reduced significantly synaptophysin labeling in hippocampal-septal projections compared to controls. These results support that, small alterations in the insulin/IGF1 pathway in specific hippocampal circuitries can underlie alterations in synaptic plasticity and affect behavior, in the absence of inflammatory conditions

Keywords Insulin resistance · IRS2 · Hippocampal dentate gyrus · Social memory · Synaptophysin · Immunofluorescence · Synaptic plasticity

Introduction

Insulin and IGF1 actions are mediated by the insulin and IGF1 receptors that can activate similar signaling pathways with pleiotropic actions in mammals. In the brain, both peptides have been shown to modulate cognitive function (Al-Delaimy et al. 2009; Cholerton et al. 2013). The signaling cascades elicited by insulin and IGF1 are almost identical and both mediated by the scaffolding proteins, the insulin receptor substrates (IRS) (Kleinridders et al. 2014; Bedse et al. 2015). There are four IRSs known isoforms, being the IRS1 and IRS2 the most abundant and studied, up to date (Bedse et al. 2015). In spite the fact that both, IRS1 and 2 have been shown to mediate insulin and IGF1 effects, some

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