



Facultad de Ciencias  
Departamento de Biología Molecular

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## **The effect of age on brain insulin signalling**

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# *ABBREVIATIONS*

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- A $\beta$ :  $\beta$ -amyloid peptide
- ABC: Atp-binding cassette transporters
- ACAT1/SOAT1: Sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1
- AChR: Acetylcholine receptor
- ACSF: Artificial cerebrospinal fluid
- AD: Alzheimer's disease
- AgRP: Agouti-related peptide
- Akt: Protein kinase B (PKB)
- AMPAR:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
- APO: Apolipoprotein
- ARC: Hypothalamic arcuate nucleus
- ATP: Adenosine triphosphate
- BBB: Blood-brain barrier
- CaMKII: Ca<sup>2+</sup> /Calmodulin-dependent Protein Kinase II
- CaRT: Cocaine and amphetamine related-transcript
- CBP: CREB-Binding Protein
- Cdk5: Cyclin dependent kinase 5
- Choox: Cholesterol oxidase
- CNS: Central nervous system
- CREB: cAMP Response Element-Binding Protein
- CSF: Cerebrospinal fluid
- CYP46A1: Cholesterol 24 hydroxylase
- DMEM: Dulbecco's Modified Eagle's Medium
- DMSO: Dimethyl sulfoxide
- ER: Endoplasmic reticulum
- Erk: Extra-cellular signal-regulated kinase
- FDA: Federal drug administration
- FRET: Fluorescence resonance energy transfer
- G0: Cell cycle resting phase
- Gab-1: GRB2-associated-binding protein 1
- GABA:  $\gamma$ -aminobutyric acid
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- GluR: Glutamate receptor (AMPA)
- GLUT: Glucose transporters
- Grb2: Growth factor receptor-bound protein 2

- GRIP: Glutamate-receptor interacting protein
- Gsk3 $\beta$ : Glycogen synthase kinase 3 beta
- HBSS: Hank's buffer salt solution
- HDAC-4: Histone deacetylase 4
- HDL: High density lipoprotein
- Hek-293T: Human embryonic kidney cells 293 T antigen transformed
- HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
- HMG-CoAR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase
- IGF-1: Insulin-like growth factor-1
- IGF-1R: Insulin-like growth factor-1 receptor
- IKK $\beta$ : I $\kappa$ B kinase  $\beta$
- IR: Insulin receptor
- IRS: Insulin receptor substrate
- IU: International units
- Jnk: c-Jun N-terminal kinase
- LDL: Low density lipoprotein
- LDLR: Low density lipoprotein receptor
- LRP1: Low density lipoprotein receptor-related protein 1
- LTD: Long-term depression
- LTP: Long-term potentiation
- LXR: Liver X receptor
- MAGUK: Membrane-associated guanylate kinases
- MARCKS: Myristoylated alanine-rich C kinase substrate
- MAPK: Mitogen-activated protein kinase
- M $\beta$ CD-Ch: Methyl- $\beta$ -cyclodextrin-cholesterol
- MCH: Melanin-concentrating hormone
- MEM: Minimum essential medium
- $\alpha$ -MSH:  $\alpha$ -Melanocyte-stimulating hormone
- mTOR: Mammalian target of rapamycin
- NMDAR: N-Methyl-D-aspartate receptors
- NPC1: Niemann-Pick disease type C1 protein
- NPC2: Niemann-Pick disease type C2 protein
- NPXpY: Binding motif for PTBs
- NPY: Neuropeptide Y
- p38: Mitogen-activated protein kinase 14 or MAPK-p38
- p70S6K: Ribosomal protein S6 kinase beta-1

- PD: Parkinson's disease
- PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
- PI(4,5)P2: Phosphatidylinositol-4,5-bisphosphate
- PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
- PKA: Protein kinase A
- PKC: Protein kinase C
- PLC $\gamma$ : Phospholipase C gamma
- POMC: Proopiomelanocortin
- PP1: Protein phosphatase 1
- PP2A: Protein phosphatase 2 A
- PP2B: Protein phosphatase 2 B
- PSD: Postsynaptic density
- PSD95: Postsynaptic density 95 protein
- PTB: Phosphotyrosine binding-domain
- PTP: Protein tyrosine phosphatase
- PVN: Paraventricular nucleus
- RTK: Receptor tyrosine kinase
- Ser: Serine
- SH2: Src homology 2 domain
- Shc: Src homology and collagen homology adaptor protein
- Shp2: SH2 domain phosphatase 2 or protein-tyrosine phosphatase 1D
- SNARE: Soluble NSF attachment protein receptor
- SREBP: Sterols regulatory element-binding protein
- T2DM: Type 2 diabetes mellitus
- Thr: Threonine
- TrkA: Tyrosine receptor kinase A
- TrkB: Tyrosine receptor kinase B
- Tyr: Tyrosine
- WB: Western blot
- VEGF: Vascular endothelial growth factor
- 24OHC: 24S-hydroxycholesterol

# *RESUMEN*

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Las vías de señalización de la insulina e IGF-1 se encuentran entre las más estudiadas en el organismo. Su regulación es crucial para el correcto funcionamiento de procesos esenciales, como la homeostasis metabólica o el desarrollo y crecimiento celular, motivo por el cual han sido estudiadas de forma exhaustiva. Sin embargo se desconocen los motivos exactos por los que sus funciones se ven alteradas ante determinadas circunstancias. Distintas publicaciones apuntan a que, además de sus numerosas funciones en todo el organismo, la insulina juega un papel importante en el cerebro en eventos tales como la modulación de la plasticidad sináptica, en particular de uno de sus subtipos conocido como *Long-term depression* (LTD). IGF-1 también ha sido relacionado con la modulación de la transmisión sináptica, aunque se desconoce el alcance de su función en ese sentido. Además la señalización de estos péptidos decae con el envejecimiento, afectando sin duda a los diferentes procesos que regulan. En esta tesis hemos estudiado el efecto de la edad sobre la señalización de insulina en hipocampo. Hemos podido determinar que la LTD dependiente de insulina se encuentra afectada en el hipocampo de ratones viejos (20-24 meses) en comparación con el de adultos (7-12 meses). Los datos bioquímicos apuntan a un exceso de actividad de los receptores, y la alta activación posterior de la cascada de señalización a nivel citoplasmático, como posible explicación del déficit funcional de esta ruta. Ésto llevaría a la activación de una retroalimentación negativa de la señalización que causaría la posterior pérdida de sensibilidad de la vía. Es importante señalar que el fenotipo observado en el hipocampo no se corresponde con signos de pérdida de sensibilidad a insulina en el resto del organismo. A nivel de mecanismo, hemos podido observar que la disminución de colesterol que ocurre de forma normal en el hipocampo de ratones envejecidos juega un papel importante en la pérdida de sensibilidad a insulina. La recuperación de los niveles de colesterol propios de ratones adultos en hipocampo de ratones envejecidos, permite restaurar la actividad basal de la vía de señalización de la insulina e IGF-1, así como una correcta LTD dependiente de insulina. Ensayos de microscopía confocal de FRET y experimentos de pérdida de función de colesterol, nos han permitido demostrar que la pérdida de colesterol que ocurre en neuronas envejecidas lleva a una activación independiente de ligando de los receptores de insulina e IGF-1, causando los efectos posteriores de pérdida de sensibilidad observados. El tratamiento con Voriconazole, un inhibidor de la enzima CYP46A1, responsable de la pérdida de colesterol en hipocampo con el envejecimiento, inhibe de forma efectiva la hiperactivación de la señalización de insulina e IGF-1 y la presencia de marcas de insensibilidad en el sustrato del receptor de insulina, permitiendo la recuperación de la señalización de la vía a niveles basales de un ratón adulto. Todos estos resultados ponen de manifiesto el papel relevante de los cambios lipídicos asociados al envejecimiento, como mecanismos efectores de la pérdida de sensibilidad a insulina vinculada a la edad en hipocampo.

# *ABSTRACT*

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The insulin and IGF-1 signalling pathways are among the most widely studied routes in the organism. Their regulation is of vital importance in processes like metabolic homeostasis or cell development and growth and therefore turned them into the object of exhaustive investigations, aiming at a deep understanding of the different processes they regulate in the different tissues of the organism. However, the intimate mechanisms by which the functions controlled by these peptides are not optimal in certain conditions remain incompletely addressed. In the brain, in addition to a body homeostasis role, a panoply of results indicate that insulin plays a role in cognition, mediated through its capacity to induce a synaptic plasticity event known as long-term depression (LTD). To a lesser extent, IGF-1 has also been related with synaptic transmission and modulation. In addition, the signalling power elicited by these peptides decreases with ageing, thus affecting the different processes they regulate. In this thesis we studied the effect of age on hippocampal insulin signalling. We determined that Insulin-LTD is impaired in the hippocampus of old mice (20-24 month-old) in comparison with adult mice (7-12 month-old). Biochemical data suggest that the functional deficit might be explained by an over-activity of the receptors leading to increased phosphorylation of the cytoplasmic effectors downstream, in turn triggering a negative signalling feedback resulting in pathway desensitisation. Importantly, the hippocampus phenotype is not accompanied by signs of abnormal body insulin sensitivity. Mechanistically, we observed that the mild reduction of cholesterol that normally occurs in the hippocampus of aged mice plays an important role in the desensitisation of the pathway in this brain structure. Restoring hippocampal cholesterol levels in old mice to the values of adult mice allows the restoration of the basal activity of the insulin/IGF-1 signalling and a proper Insulin-LTD. Through FRET imaging and cholesterol loss of function experiments we demonstrate that the cholesterol loss occurring in old neurons leads to ligand-independent activation of the IR and the IGF-1R, consequently driving the posterior desensitising effects observed. The treatment with Voriconazole, an inhibitor of the CYP46A1 enzyme, responsible for the cholesterol loss in old mice hippocampus, effectively avoids the hyper-activation of the IR/IGF-1R signalling and the presence of insulin desensitisation marks on the IR substrate, allowing the return of the pathway to an adult basal state. All together these results put age-associated changes in lipid content at a most upstream mechanism of age-associated hippocampal insulin desensitisation.

# *INTRODUCTION*

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The ageing process is defined as the time-dependent decline of biological properties that affects all organisms, impairing physical and cognitive functions. Despite it is not a disease by itself, ageing is a pro-morbid state that predisposes the organism to severe pathologies, including cancer and neurodegenerative disorders. Considering that in the next 30-40 years the number of people over 60 years old will have doubled, elucidating the mechanisms of the different processes that drive ageing appears an important issue in the biomedical field. Nowadays, there is consensus about the different features of the ageing process, constituting what are now known as the “Hallmarks of ageing” (López-Otín et al., 2013). These are: Genomic instability (mainly represented by DNA damage), telomere attrition, epigenetic alterations (that modify gene expression patterns), loss of proteostasis (affecting proteins’ folding, function and degradation), impaired nutrient sensing (altering principally insulin/IGF-1 signalling), mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication comprise the main characteristics of ageing (López-Otín et al., 2013). Several of the events that lead to the ageing phenotype are regular processes that occur physiologically in the cells in the course of every step in life, from early development to maturity (inflammatory/anti-inflammatory response or cellular senescence), however different defects and cellular insults that accumulate in time could turn these cell responses into deleterious effects that finally drive the functional decay of the old. Likewise the exacerbated expression of some of these normal ageing characteristics has been linked to the occurrence of degenerative diseases (e.g. macular degeneration, Alzheimer’s disease).

As the rest of tissues in the organism, the brain also manifests the same ageing hallmarks, though adapted to its particular properties; namely that neurons, key cells in the cognitive and motor functions, become permanently arrested in the G0 phase of the cell cycle in the early stages of life, and thus must remain alive for the entire lifespan of the individual in order to guarantee proper communication. As neuron-neuron and neuron-target cell communication reveals essential in brain nature, the incapacity of neurons to undergo replacement makes these cells especially vulnerable to the effects of time. Thus, in the ageing brain synaptic loss and synaptic plasticity impairments are the main characteristics, more than the loss of neurons. This phenotype is indicative that synaptic function and not survival function is the main target of the ageing process in the brain. On the other hand, neuronal death is representative of pathological states like Alzheimer’s or Parkinson’s disease (Morrison et al., 2012).

Numerous brain structures participate in the cognitive process, both subcortical (the hippocampus, cerebellum, amygdala and basal ganglia) and cortical (frontal, parietal, oc-

capital and temporal lobes) structures. Thus, cognitive decay in the old must involve communication deficits at all these levels. The most comprehensively studied of these structures is the hippocampus, which plays an active role in memory storage and the formation of new memories. We will summarise in this introduction the principal signalling events that characterise the synaptic functions in the hippocampus and how a reduced insulin/IGF-1 signalling, a constitutive defect in the old organism (Fontana et al., 2010; Mattison et al., 2012), could lead to cognitive deficits of the old. In addition, we will refer to the changes in brain cholesterol metabolism that occur during ageing, which we envision may be an upstream determinant of synaptic and insulin signalling defects of the old.

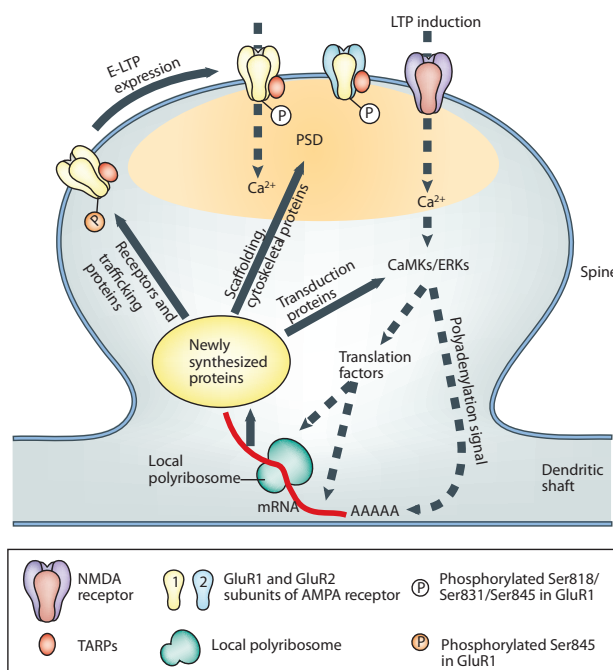
### 1. Synaptic plasticity

One of the most interesting and important properties of the mammalian brain is its capacity to incorporate and retain external signals and experiences, in what is usually known as learning and memory processes. In its simplest form, learning and memory imply the transformation by neurons of incoming chemical signals into electrical currents, which are then integrated into neural circuits. The capacity of modifying these neural circuits, modulating, consolidating or removing them, to finally generate thoughts, feelings, memories and behaviours, is known as synaptic plasticity. More technically, synaptic plasticity comprises the modulation of strength and efficiency of the electric signals in synapses already established. The modifications of electric signals in synapses refer to any process that enhances or depresses them and lasts in time, allowing these processes to generate signalling patterns that induce a change sufficient to create a new knowledge (learning) that can persist, as for example long-term memories. These alterations occur at the molecular and structural level both locally (at the synapse and peri-synaptic space) but also distantly, in the cell body and the nucleus, being different signalling pathways implicated. In these processes not only neuronal (synaptic) terminals participate but also the surrounding astrocytes (see Navarrete et al., 2012; Perea et al., 2014).

The two best-characterised cellular processes underlying synaptic plasticity are long-term potentiation (LTP), referring to the persistent increase in synaptic strength following high frequency stimulation, and Long-term depression (LTD), an activity-dependent reduction in synaptic strength following a long patterned stimulus. In addition to patterned stimuli to elicit LTP and LTD, there are other means to induce them, each with particular characteristics (Citri et al., 2008). Here we will summarise the pharmacological (NMDA-dependent) form of LTP and LTD and the specific subtype that will be studied in this thesis: Insulin-LTD.

### 1.1 NMDAR dependent long-term potentiation

Initially observed in the CA1 region of the hippocampus, LTP is a kind of synaptic plasticity triggered by activation of N-Methyl-D-aspartate receptors (NMDARs), and influenced by posterior modulation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA) (Martin et al., 2000). These receptors are ionotropic glutamate receptors present in the postsynaptic region of the synapse (Figure I1). NMDARs are permeable to  $\text{Ca}^{2+}$  and  $\text{Na}^+$  and their activation following the release from presynaptic terminals of the neurotransmitter glutamate, which binds NMDARs (Nicoll et al., 1988), leads to the opening of the channels and membrane depolarisation. This initial depolarisation is propagated, in the plane of the membrane towards the cell body, to sub-synaptic domains to regulate receptor density at the plasma membrane, and towards the nucleus for the regulation of gene expression. During basal synaptic activity AMPARs provide most of the inward current, while NMDARs remain blocked by extracellular  $\text{Mg}^{2+}$  at negative membrane potentials (Mayer et al., 1984). A strong depolarisation is needed to unblock NMDARs channels, allowing an important inward current of  $\text{Ca}^{2+}$  that is required to reach the threshold for activating the signals necessary for achieving the receptor number change needed for LTP (Malenka, 1991).



**Figure I1. Long-term potentiation processes.**

The figure represents the main LTP molecular processes. Initial inward  $\text{Ca}^{2+}$  currents through NMDARs channels activate CaMKII and other kinases. This activation leads to biosynthesis of new proteins and modification in pre-existing scaffolding proteins, channels and regulatory proteins. All these events cause an increase in AMPARs delivery to the membrane and their attachment to the postsynaptic density, favouring an intensification of synaptic signaling, and thus promoting the maintenance of the LTP in time. Adapted from Derkach et al., 2007.

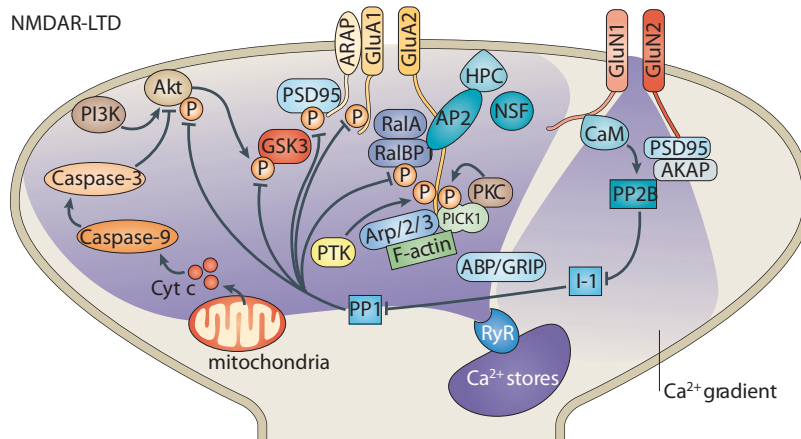
Once NMDARs channels are opened and  $\text{Ca}^{2+}$  enters the cell, different biochemical changes occur. The most relevant is the activation by autophosphorylation of the calcium/calmodulin-dependent protein kinase II (CaMKII), an essential step for LTP (Malinow et al., 1989; Pettit et al., 1994; Giese et al., 1998). Other kinases are also activated as result of the

initial triggering and contribute to LTP. Thus, via PKA activity the activation of inhibitor 1 occurs, which inhibits protein phosphatase 1 (PP1) avoiding PP1-mediated dephosphorylation of CaMKII (Makhinson et al., 1999). PKC or MAPK/Erk (Erk) have also been described to be important for later maintenance of LTP (Thomas et al., 2004; Serrano et al., 2005). However, the decisive event in LTP is the increase in AMPAR trafficking and insertion in the plasma membrane by the activation of these kinases (Derkach et al., 2007): CaMKII-mediated phosphorylation of scaffolding proteins to favour receptor insertion (Tomita et al., 2005) and PKA and PKC inducing AMPARs phosphorylation (Esteban et al., 2003; Boehm et al., 2006). LTP maintenance requires receptor insertion at the peri-synaptic membrane followed by lateral diffusion to the postsynaptic density (PSD). This is an area located in the postsynaptic terminal, composed by multiple elements (some of the best characterised are PSD95, Stargazin, MAGUK protein family) that in the end determine the proper architecture and function of the synapse, including the insertion and retention of AMPARs and as consequence the sustaining of the signal (Citri et al., 2008). In addition to AMPA receptor insertion, the persistence in time of LTP also relays in changes in dendritic spine's architecture (Lüscher et al., 2000), and in local dendritic protein synthesis (Sutton et al., 2006) that would provide with components for the long-term maintenance of synaptic strength, in what is called late-phase of LTP.

### **1.2 NMDAR dependent long-term depression**

Conversely to LTP, LTD is based on the weakening of the synaptic signals (Dudek et al., 1992). LTD discovery settled the theory that encoding of learning and memories in the brain relies on the modulation and distribution of synaptic weights in complex neural circuits (Mulkey et al., 1992) and not only in enhancer signals. As LTP, LTD depends on an initial activation of NMDARs, thus requiring an inward  $\text{Ca}^{2+}$  current. The main difference consists in the strength of the stimulation, which is weaker in LTD. However it must be enough for partially relieving the  $\text{Mg}^{2+}$  block of NMDARs (Selig et al., 1995). Once the entry of  $\text{Ca}^{2+}$  is initiated, the signalling cascade differs from LTP (Figure I2). If kinases were the main figures in LTP, phosphatases gain relevance in LTD. A slight (compared to LTP)  $\text{Ca}^{2+}$  entry leads to calcium/calmodulin-dependent activation of the phosphatase calcineurin, also known as protein phosphatase 2B (PP2B), different from the effect of larger amounts of  $\text{Ca}^{2+}$  which result in the activation of CaMKII and LTP (Winder et al., 2001). Once activated, PP2B inhibits the protein inhibitor 1 through its dephosphorylation, thus avoiding PP1 repression and facilitating CaMKII dephosphorylation and inhibition (Lisman et al., 1989; Strack et al., 1997). In addition, other phosphatases like PP1 and protein phosphatase 2A (PP2A) become active further guaranteeing the achievement of LTD (Mulkey et al., 1993). All in all, with phosphatases activation during LTD, not only CaMKII but also other kinases involved in LTP such

as PKA or PKC become dephosphorylated (Lee et al., 2000) and inactivated. Likewise, dephosphorylation also affects the AMPARs residues that potentiate their conductance (Lee et al., 2000). This situation, together with the activation and inhibition of different proteins of the postsynaptic density or proteins implicated in lateral diffusion and architecture of the cell (Carroll et al., 2001), finally lead to the dissociation of AMPARs from the postsynaptic density and their endocytosis, in the end producing a weakening in synaptic strength.



**Figure 12. Long-term depression processes.**

The image shows the main LTD molecular processes. Lower initial  $\text{Ca}^{2+}$  currents activate PP2B instead of CaMKII. This situation leads to the activation of different phosphatases (PP1, PP2A) that inhibit the proteins that normally become active upon LTP. Phosphatases signalling is also driving modifications in scaffolding proteins of the postsynaptic

density that allow AMPARs to detach and be laterally mobilised heading to its endocytosis. Other proteins that were inactive under LTP signalling, like Gsk3 $\beta$ , are dephosphorylated and activated by phosphatases, enhancing AMPARs endocytosis and thus allowing signalling depression. Adapted from Collingridge et al., 2010.

Although the role of phosphatases seems predominant in LTD, several kinases become active in this signalling as well and develop essential processes for the achievement of the synaptic depression. This is the case of proteins like cyclin-dependent kinase 5 (Cdk5) (Ohshima et al., 2005), p38 (Zhu et al., 2002) or Gsk3 $\beta$ . Gsk3 $\beta$  in particular has revealed as an important character for AMPARs endocytosis, being inhibited by Akt phosphorylation under LTP, and activated by PP1 dephosphorylation when LTD is initiated. Its blockage prevents LTD induction (Peineau et al., 2007), representing a mechanism through which a synaptic plasticity type impedes the other. Finally LTD is also maintained in time by means of changes in spine morphology (Zhou et al., 2004) and protein synthesis (Pfeiffer et al., 2006).

### 1.3 Non-NMDA synaptic plasticity: the role of insulin

In the mammalian brain, different classes of long-term potentiation and long-term depression events take place, being enhancement or decline determined by the type of triggering stimulus, its intensity, presence of other stimuli, age, metabolic and stress background, all in all defining the different learning and memory experiences (Citri et al., 2008). Numerous

works indicate that insulin contributes to these changes. Thus, the observation of high concentrations of insulin receptor in the hippocampus, one of the brain structures with a crucial function in spatial learning and memory, was one of the first evidences supporting a role of this peptide in learning and memory (Wickelgren, 1998; Sonntag et al., 2005). This notion was later confirmed by a number of functional studies in rodents, showing that central insulin is involved in cognitive processes such as attention and executive functioning (Zhao et al., 1999; Kern et al., 2001). Moreover, also in humans this role found support, especially from the works that showed that the direct application of insulin to the central nervous system (CNS) was an effective mean to reduce cognitive decline with age and also in Alzheimer's disease (AD), and to also improve memory in young adults (Benedict et al., 2007; Freiherr et al., 2013). Mechanistically, these functional observations were supported by a number of recent evidences, among which are worth mentioning those that showed that insulin contributes, as co-adjuvant and even directly, to LTP and LTD (Van Der Heide et al., 2005; Martin et al., 2012; Blázquez et al., 2014). Before getting into the details of how brain insulin contributes to synaptic plasticity, in the following sections we will summarise some key features of insulin signalling in general, both in the control of body homeostasis and in brain function.

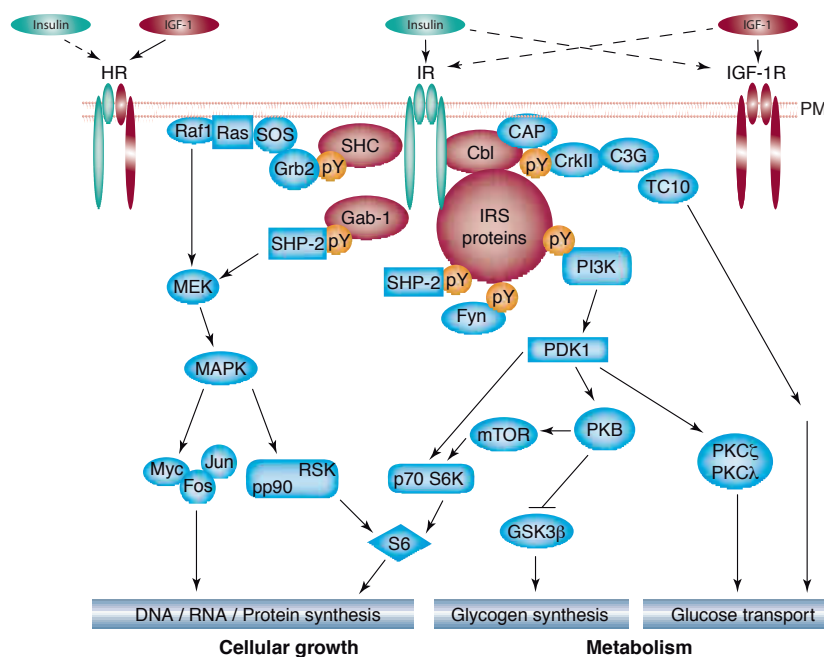
## 2. Insulin and IGF-1 signalling

Insulin and insulin-like growth factor-1 (IGF-1) are some of the peptides/hormones more extensively studied in molecular biology, exerting their functions along all tissues and cells. In addition to its specific role in the regulation of energetic homeostasis of all body cells, insulin is also implicated in cell division and development (Blázquez et al., 2014). IGF-1 signalling has been mainly attributed a mitogenic role, with important functions in cell division and differentiation, taking relevance in cell maintenance, survival and tissue repair (Fernandez et al., 2012). In the brain, the best-studied role of insulin is on the control of food intake and satiety although lately both peptides have been attributed an important role in neuronal signalling and synaptic plasticity (Huang et al., 2004). Importantly, they have also been linked with organismal ageing, with age-associated signalling down-regulation being considered as a physiological response to ageing in order to increase lifespan. In the following sections we will summarise insulin and IGF-1 signalling pathways and their roles in the CNS.

### 2.1 *Insulin and IGF-1 mechanisms of action*

Insulin and IGF-1 function require binding to their respective receptors. Insulin receptor (IR) and IGF-1 receptor (IGF-1R) belong to the receptor tyrosine kinase (RTK) family. Their structures are very similar and they share downstream signalling proteins, often acting cooperatively (Fernandez et al., 2012). The fact that both pathways are so intimately related

indicates that alterations in one route could influence on the other's performance (Yakar et al., 2001; Moloney et al., 2010). Both types of receptors are formed by two  $\alpha$ -subunits (extra-cellular domain), each of which is bound by disulphide bonds to a  $\beta$ -subunit (transmembrane and cytosolic domains), forming  $\alpha\beta$  homodimers (O'Neill, et al., 2012). Although each receptor can bind either peptide (i.e. IR can bind IGF-1 and vice-versa) the affinity is far higher for its own ligand. Moreover the  $\alpha$ - $\beta$  dimers can dimerise with subunits from the other type of receptor, giving rise to hybrid receptors that can bind both peptides, with preference for IGF-1 rather than insulin (Pandini et al., 2002).



**Figure I3. Insulin/IGF-1 receptors and signalling cascades.**

The picture depicts a simplified version of insulin/IGF-1 intracellular signalling pathways, using IR cascade as general example. IR and IGF-1R are highly homologous tyrosine kinase receptors. They can form hybrid receptors (HR) and bind each other ligand with lower affinity. The ligand induces cytosolic-tail auto-phosphorylation activating downstream proteins like IRS, SHC, Gab-1 or Cbl, which act as docking sites for different molecular effectors. This signalling activates two of the main

pathways for cell growth and survival: PI3K/Akt and MAPK/Erk pathways. Adapted from Zick et al., 2001.

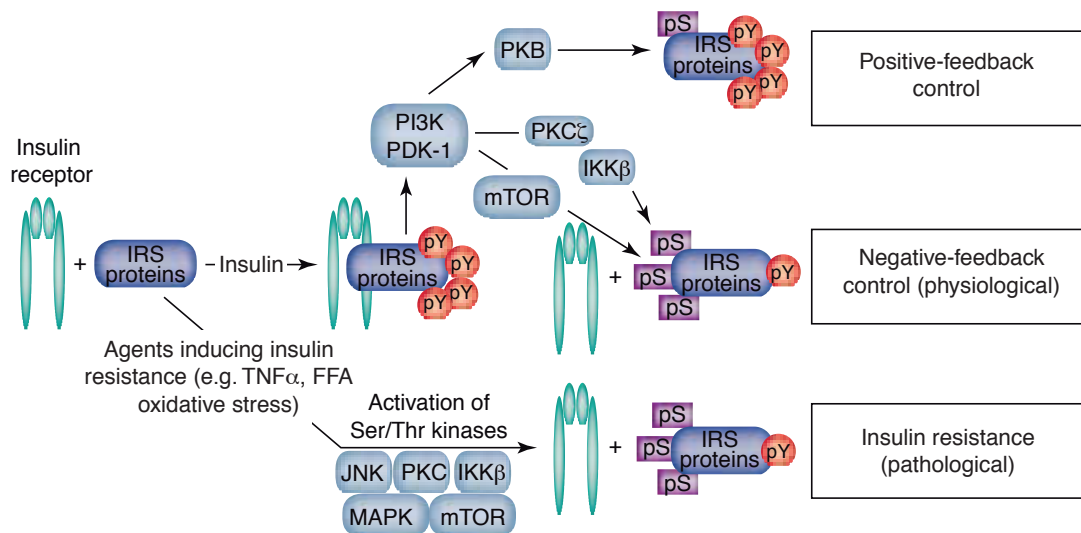
To a large extent, ligand-receptor binding for both peptides elicits similar downstream signalling events (Figure I3). Ligand binding to the extracellular domain induces a structural modification in the transmembrane-region that drives this domain approximation and cytosolic-domain approximation and autophosphorylation at tyrosine residues, in turn triggering the phosphorylation of tyrosine residues in downstream effectors (Hubbard et al., 1994; Taguchi et al., 2008; Kavran et al., 2014). These belong to two main pathways: PI3K and Erk. PI3K activation occurs by the docking to insulin receptor substrate scaffolding proteins (IRS 1 to 4) following their activation by IR/IGF-1R phosphorylation. Erk is also activated after IR/IGF-1R phosphorylation though through adaptors like Shc/Grb2 or Gab-1/Shp2 (Zick, 2001). In general terms, PI3K pathway activation by insulin/IGF-1 is more related to cell survival and metabolic regulation and Erk activation to mitogenic stimuli, survival and differentiation.

Likewise IGF-1R has been detected in the nucleus (Sehat et al., 2010), where it is thought to affect gene transcription directly. This last fact and the different receptors' spatial and temporal expression patterns, transcriptional regulation and cellular compartmentalisation, could explain the existence of different functions for these receptors despite having the same downstream signalling pathway.

Different control mechanisms have evolved to regulate the intensity and duration of the signals elicited by ligand binding, enhancing or attenuating them according to cell requirements. The IRS and Shc proteins are docking proteins playing a key role in propagating insulin/IGF-1 signalling (Paz et al., 1997; Zick et al., 2001). These proteins contain a phosphotyrosine binding-domain (PTB-domain) which binds to the NPXpY motif in the insulin/IGF-1 receptor. The subsequent phosphorylation on IRS creates binding sites for the SH2 domains of multiple signalling proteins, responsible for the recruitment and activation of the intracellular PI3K/Akt and MAPK/Erk biochemical pathways. Some differences exist however between IRS and Shc binding. The PTB domains of Shc and IRS both recognise autophosphorylation sites in RTKs but show distinct abilities to bind to different RTKs (Eck et al., 1996), such as the TrkA nerve growth factor receptor and the insulin receptor. Some studies suggest that subtle differences in the types of residues N-terminal to the NPXpY motif may determine the affinity with which phosphopeptide ligands are recognised by the Shc and IRS PTB domains. *In vitro* experiments have shown that, unlike IRS-1, the Shc PTB domain binds poorly to the IR beta subunit, owing to its low affinity for the NPXpY autophosphorylation site at Tyr 960 (Van Der Geer et al., 1996) As a consequence, Shc does not bind stably to the activated IR in certain cells. This type of mechanism may explain the different trajectories of insulin and IGF-1 signalling in different cells, to direct signalling more or less towards the PI3K or MAPK cascades.

Signalling termination occurs upon the activation of particular proteins downstream in the pathway, however unrelated proteins are also able to drive it, like protein tyrosine phosphatases (PTPs) that can dephosphorylate the receptor or the downstream activated proteins, thus inactivating them (Elchebly et al., 1999). In either case, the main physiological termination event conforms a negative feedback that allows the reduction of the signalling once the functions are accomplished (e.g. when blood insulin or glucose levels start to decrease). One of the stronger manners to attenuate IR/IGF-1R signalling is through Serine/Threonine (Ser/Thr) phosphorylation on the IRS adaptors. There are several Ser/Thr residues in the IRS that could be considered as regulatory marks, enabling multiple control levels in a pathway that needs a complex and tight regulation. These marks usually disrupt the in-

ulin/IGF-1 activation signalling at IRS level (Zick et al., 2001).



**Figure I4. IRS protein-mediated control feedbacks.**

The image shows the different control feedbacks mediated by IRS proteins phosphorylation. Akt/PKB exerts a positive regulation by IRS serine phosphorylation, protecting IRS proteins from phosphatases action. mTOR, p70S6K and PKC/IKK $\beta$  regulate the physiological negative feedback of the pathway, allowing a correct performance of the IR/IGF-1R signalling and avoiding deleterious over-activating effects. Aberrant signalling, chronic inflammation and other altered stimuli cause a pathological negative feedback that leads to an insulin resistance state and disease (Type 2 diabetes mellitus, AD...). Adapted from Zick et al., 2001.

Negative phosphorylation of IRSs is largely due to mTOR, p70S6K and PKC, downstream of PI3K/Akt pathway (Liu et al., 2001; Yuan, 2001). Negative Ser307, Ser616 or Ser632 on IRS-1 are the consequence of the activity of these kinases in rodents (also in the homologous residues in humans), being p70S6K a strong regulator of Ser307 and Ser632 phosphorylation (Shah et al., 2004; Um et al., 2004). Other proteins like Jnk could also perform some of these regulatory marks, being Ser307 linked with this and other pro-inflammatory proteins like IKK $\beta$  (Kanety et al., 1995; Aguirre et al., 2000; Yuan et al., 2001; Figure I4). The aberrant activation of all these kinases under certain pathological or stress conditions, like oxidative stress or chronic inflammation, leads to a permanent desensitised state called insulin/IGF-1 resistance, that impairs ligand signalling contributing to the development of different diseases (obesity, Type 2 diabetes, AD, etc.).

## **2.2 Insulin and IGF-1 functions**

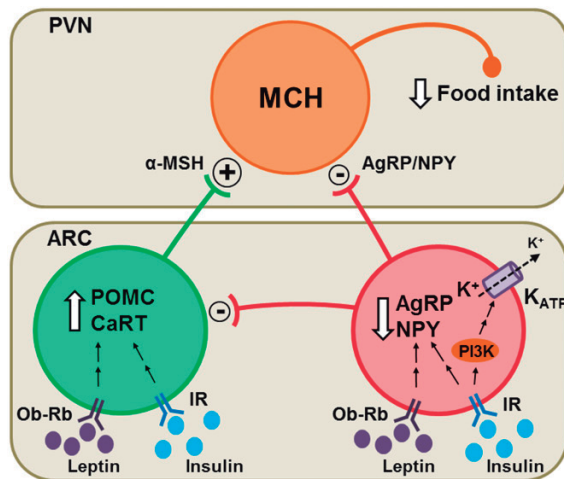
### *2.2.1 Insulin physiological roles:*

The main function of insulin in peripheral organs is the maintenance of glucose homeostasis, contributing to glucose uptake in almost all the tissues and with special importance in those in charge of nutrients storage like liver, adipose tissue and muscle. Briefly, insulin is synthesised in the  $\beta$  cells of the pancreatic islets of Langerhans and stored in vesicles until release. Following food intake, glucose enters  $\beta$  cells freely by GLUT-2 (glucose transporter 2) channels, and its degradation generates ATP. The increment in cellular ATP levels induces  $K^+$ -ATP channels closure, resulting in depolarisation of the cell and activation of voltage dependent  $Ca^{2+}$ -channels. Inward  $Ca^{2+}$  leads to vesicle exocytosis and insulin release (Wilcox, 2005). Different factors could influence  $\beta$  cells depolarisation and insulin secretion. Signals from hypothalamus (cholinergic stimulating, adrenergic inhibiting) (Wilcox, 2005), incretin hormones from the gut (like GLP-1 or GIP both stimulating) (Drucker et al., 2006) and amino acids or fatty acids (both activating) could influence insulin secretion. Insulin-receptor association favours glucose transport, mainly through GLUT-4 insertion at the cell membrane, allowing glucose to entry. Likewise, insulin triggers glycogen synthesis in liver and muscle, gluconeogenesis inhibition in liver, fatty acid and cholesterol synthesis induction and lipolysis inhibition in adipose tissue. All in all, insulin's principal role is a shift from catabolic to anabolic processes, required for cell growth, differentiation and energetic homeostasis effects in different tissues (Wilcox, 2005).

### *2.2.2 Insulin in the brain:*

During years, the brain was considered an insulin-insensitive organ, due to absence of insulin regulation for glucose uptake. However, we now know that insulin performs other functions in the brain, like the body's glucose homeostasis and feeding behaviour control, neuronal development and learning and memory. In order to satisfy the demand of glucose brain cells utilise specific transporters independently from insulin action: GLUT-1 (endothelial cells and astrocytes), GLUT-2 (neurons in the hypothalamus), GLUT-3 (neurons of cerebellum, cortex, striatum and hippocampus, observed also in glial and endothelial cells), GLUT-4 (olfactory bulb, hippocampus, hypothalamus and cortex at low levels, slightly regulated by insulin) and GLUT-8 (glucose internal release in neurons). These transporters ensure glucose supply for the CNS, completely essential for its function, thus enabling insulin to develop other functions (Gould et al., 1992; Piroli et al., 2002; Talbot et al., 2012; Blázquez et al., 2014). Brain insulin comes from different sources. On the one hand it reaches the brain from the plasma through the blood-brain barrier (BBB), a structure that isolates the cerebrospinal

fluid (CSF) of the circulating blood, through a saturable transport process (Miller et al., 1994). It was also proposed that insulin could also access CNS via some regions like the hypothalamus where the BBB shows a laxer conformation (Kleinridders et al., 2013). The third source of brain insulin is by local synthesis. Although this possibility is still under debate, neurons not only synthesise insulin but also release it under certain stimuli like depolarising signals (Wei et al., 1990). Nevertheless the rate of insulin synthesis is lower in brain than in the periphery (Lee et al., 2016).



**Figure I5. Insulin regulation in the hypothalamus.**

The picture shows the regulation of food intake driven by insulin in the hypothalamus. Insulin exerts its effects in orexigenic and anorexigenic neurons of the hypothalamic arcuate nucleus (ARC). It induces the downregulation of neuropeptide Y (NPY) and agouti-related peptide (AgRP) synthesis, two peptides that avoid satiating signals, together with hyperpolarisation that blocks the orexigenic neurons signalling to the paraventricular nucleus (PVN). Both events abrogate the food intake stimulation. At the same time insulin induces the transcription of proopiomelanocortin (POMC) and cocaine and amphetamine related-transcript (CaRT) in anorexigenic neurons. These peptides induce the synthesis of

$\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) that acts on melanin-concentrating hormone (MCH) neurons in the PVN of the hypothalamus leading to signals that reduce food intake. Adapted from Kleinridders et al., 2014.

As mentioned, insulin exerts different functions in the brain, many of which are region-specific. Thus, in the hypothalamus, insulin seems to be mainly involved in the control of feeding behaviour and energy homeostasis. In this structure, arcuate nucleus (ARC) neurons sense blood insulin levels and produce diverse responses: orexigenic neurons expressing peptides like neuropeptide Y (NPY) or agouti-related peptide (AgRP) favour food intake whereas anorexigenic neurons expressing peptides like proopiomelanocortin (POMC) and cocaine and amphetamine related-transcript (CaRT) decrease food intake (Kleinridders et al., 2014). The insulin signalling induces in orexigenic neurons the reduction of both NPY and ArRP synthesis thus decreasing food intake stimulating signals. Simultaneously insulin leads to  $K^+$ -ATP dependent channels opening in orexigenic neurons, causing the hyperpolarisation of the cells (Figure I5). This hyperpolarised status blocks their capacity to transmit signals to neurons in the paraventricular nucleus (PVN) where finally stimuli for food intake are triggered or not (Spanswick et al., 2000; Plum et al., 2005). At the same time insulin induces synthesis in anorexigenic neurons of POMC and CaRT, leading to  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) production (Benoit et al., 2002; Steculorum et al., 2014). This hormone has

its receptors MC4R in the PVN neurons, driving their activation and allowing them to signal for reducing food intake (Schwartz et al., 1996). Absence of insulin together with low levels of blood glucose triggers the opposite signalling, leading to food intake. It is considered that the obesity observed in pathologic situations like insulin resistance may be in part explained by the disruption of the orexigenic-anorexigenic signalling cascades (Kleinridders et al., 2009).

Outside the hypothalamus, insulin's role has been linked to different steps during brain development and growth. Thus, early work demonstrated that IR number increases during early stage of development (Roger et al., 1980) and ulterior work proved that this is an important event during the process of cell generation and for differentiation. Recent work also demonstrated that insulin is important for the survival and maintenance of neural stem cells (Rhee et al., 2013). In addition to its role in differentiation, insulin was demonstrated to be a potent neuroprotective agent, acting against processes like oxidative stress, beta amyloid toxicity and apoptosis (Ryu et al., 1999; Rensink et al., 2004). Besides, insulin was shown to protect neurons against injury damages, e.g. the effects caused by ischemic lesions can be reduced by insulin administration (Shuaib et al., 1995). In the hippocampus, insulin was shown to regulate GABA receptor density in postsynaptic domains, thus modulating electric signalling (Wang et al., 2003). It was also shown to participate in the uptake of amino acids required for synthesis of neurotransmitters and, as early mentioned, to take part of synaptic plasticity events (described in detail below).

### *2.2.3 IGF-1 signalling roles:*

IGF-1 peptide is involved in cell cycle, survival, development and differentiation all across the body. This peptide is produced mainly in the liver, however all cells in the brain can express it. In addition to local synthesis, this peptide can reach the brain from the blood through IGF-1R expressed at the BBB (Nishijima et al., 2010), which seems to show higher permeability to IGF-1 than to insulin. Another group of proteins, IGF binding proteins (IGFBPs), bind IGF-1 in the body and in the CNS regulating the bioavailability of the IGF-1 peptide. This group of proteins allows IGF-1 to prolong its half-life and are involved in processes like transport from blood to the CSF through the BBB (Fernandez et al., 2012).

Regarding its functions, IGF-1 is implicated mainly in cell growth and differentiation, being even linked with aberrant cell growth processes like tumour development (Le Coz et al., 2016). At the brain level, IGF-1 is especially important in the development of CNS, for both neuronal and glial cells. Consistently, lack of IGF-1 causes alterations in brain growth and severe functional defects (Liu et al., 2009). In the adult brain IGF-1 has also relevant

roles in cell survival, principally after brain injuries, e.g. ischemia (Walter et al., 1997; Beilharz et al., 1998). As insulin, IGF-1 also plays a role in neuronal plasticity, due to its capacity to induce synaptic responses like LTD in the cerebellum (Wang et al., 2000) and improving cognitive tasks. Additionally, IGF-1 was shown to play an essential role in the formation of new neurons in adult brain and contributing to the recovery of learning and memory tasks after lesions (Trejo et al., 2001).

#### *2.2.4 Insulin and synaptic plasticity:*

Several studies demonstrated the importance of insulin and insulin-signalling on electrically induced long-term potentiation (LTP). For example, haploinsufficiency of the IR  $\beta$ -subunit (Nisticò et al., 2012) or disruption of downstream signalling steps like IRS-proteins were found to impair CA1-LTP in the hippocampus (Martin et al., 2012). On the same line, it was shown that insulin treatment prevents the impairment in hippocampal CA1-LTP induced by streptozotocin in a rat model of diabetes (Izumi et al., 2003). Moreover, other studies reported that infusion of glucagon-like peptide 1 (GLP-1) and enzymatically stable analogues promote insulin sensitivity and to either enhance LTP under control conditions (Gault et al., 2008) or to ameliorate the deficits in LTP seen in different animal models of diabetes (Lennox et al., 2014). Thus, insulin modulates or promotes hippocampal LTP.

In addition to the function of insulin on LTP, insulin has a core mechanistic role in long-term depression (LTD). Thus, the mere application of insulin was found to cause LTD in the hippocampus without the necessity of additional electrical stimulation (Man et al., 2000; Huang et al., 2004; Van Der Heide et al., 2005). According to these studies, hippocampal insulin-induced LTD (Insulin-LTD) is (i) generated by an insulin-induced AMPAR-internalisation (Man et al., 2000; Ahmadian et al., 2004), (ii) depends on an increase of postsynaptic  $\text{Ca}^{2+}$  concentration which is fed by either  $\text{Ca}^{2+}$ -influx via NMDA receptors, L-type voltage-activated  $\text{Ca}^{2+}$  channels and the release of  $\text{Ca}^{2+}$  from intracellular stores, respectively, (iii) the activation of the PI3K/Akt pathway and (iv) the rapamycin-sensitive local translation of dendritic mRNA (Huang et al., 2004). Altogether, these changes drive local changes in the scaffolding and structural proteins at the PSD leading to AMPARs endocytosis. These are the major properties of this type of LTD.

### **2.3 Insulin and IGF-1 in brain ageing and disease**

One pathway that is coming more and more into focus as a regulator of cognitive decline during ageing is the insulin/IGF-1 pathway. Clinical and preclinical studies revealed a reduction of insulin and IGF-1 receptors, their message and their function in the hippocampus

during pathological ageing (Frölich et al., 1998; Zaia et al., 2000; Zhao et al., 2004; Deak, et al., 2012; De Felice et al., 2014; Talbot et al., 2014; Chami et al., 2016). A similar down-regulation of brain insulin receptor signalling reduction was found in animal models of ageing and AD, often accompanying peripheral metabolic deregulation in type 2 diabetes mellitus (T2DM) (Pedersen et al., 2004; Rowe et al., 2007; Stranahan et al., 2008; Zhao et al., 2008; McNay et al., 2011). It is currently thought that the reduced insulin signalling that occurs in this organ during pathological ageing may be due to mechanisms analogous to those that account for peripheral insulin resistance in T2DM (Ferreira et al., 2014).

As we have previously mentioned, both insulin and IGF-1 peptides are important for cell growth and tissue development. Their receptors are widely expressed in rodents during development. Insulin receptors are found in olfactory bulb, hypothalamus, sub-thalamic nucleus, sub-fornical organ, CA 1/2 pyramidal cell layer of the hippocampus, cerebellum, amygdala, and cerebral cortex (Unger et al., 1989). Likewise IGF-1R is expressed in cortex, hippocampus, cerebellum, hypothalamus, brainstem and spinal cord (Adamo et al., 1989, Fernandez et al., 2012). During normal ageing these patterns seem to change, however there is controversy whether there is a decline or not in these signalling pathways. Different publications have reported a decrease in mRNA transcription levels of insulin/IR and IGF-1/IGF-1R pathways in rodents and in humans, in agreement with the works that reported decrease at the protein level (D'Costa et al., 1995; Frölich et al., 1998; Lee et al., 2014). Contrarily, other researchers showed lack of changes or even increased levels with age (Doré et al., 1997; Chung et al., 2002). Though it seems that the loss of activation of these receptors with normal ageing is more accepted (Fernandes et al., 2001; Poe et al., 2001), leading to a reduction in the capacity of these signalling cascades to become active upon stimuli.

Moreover, in many instances insulin and IGF-1 resistance develop in parallel with conditions associated to inflammation and oxidative stress that finally could drive a worsening in the normal ageing phenotype and the development of diseases like T2DM and AD. Numerous evidences indicate that both pathologies are closely connected. Thus, it was observed that hyperinsulinemia and hyperglycaemia due to insulin resistance enhance neuritic plaques formation (Matsuzaki et al., 2010) and in turn such A $\beta$  deposition reinforces brain insulin resistance through IR signalling reduction (Xie et al., 2002; Lee et al., 2009). Likewise situations of chronic inflammation, oxidative stress and insulin resistance linked to obesity and T2DM could favour A $\beta$  deposition (Sheng et al., 2003; Nizari et al., 2016). Ultimately, A $\beta$  and insulin are both substrates of insulin-degrading enzyme, and it was shown that A $\beta$  degradation is impaired in T2DM hyperinsulinemia situations by the competitive blockage of the de-

grading enzyme (Farris et al., 2003). Thus the association insulin resistance with pathological brain ageing is not only supported by different epidemiological studies in humans but also by biochemical cause-effect evidences in different experimental set-ups.

In light of the above association, different treatments have emerged lately to compensate this insulin/IGF-1 signalling impairment and ameliorate Alzheimer's symptoms. Therapies that stimulate insulin secretion or intranasal delivery of insulin/IGF-1 have been demonstrated to decrease A $\beta$  deposition in mice and humans (Carro et al., 2002; Craft et al., 2012). Similarly, treatments in mice and humans with insulin sensitising agents like metformin or incretin analogue molecules have demonstrated to be effective in the treatment of insulin resistance, reducing mild cognitive impairment (Hsu et al., 2011; McClean et al., 2011). On the other hand, numerous publications indicate that insulin/IGF-1 signalling reduction in different species is associated with longer lifespan, and better outcome of Alzheimer's (Holzenberger et al., 2003; Van Heemst et al., 2005; Selman et al., 2008). Thus, it was shown that the partial ablation of IGF-1R in mice models for AD reversed its symptoms through decreasing soluble A $\beta$  oligomers and reducing inflammation (Cohen et al., 2009). Hence, although all these data clearly indicate that the brain is, like any other organ, highly sensitive to insulin and IGF-1 signalling, we still do not know the intimate mechanisms involved in brain insulin/IGF-1 signalling reduction with age, and even less how these affect brain pathological conditions associated with ageing like AD. In this regard, it appears important to mention the numerous studies reporting that the membranes of erythrocytes, leukocytes and skeletal muscle cells of diabetics are rich in rigidity-promoting lipids, namely cholesterol, sphingomyelin and saturated fatty acids (Borkman et al., 1993; Ruiz-Gutierrez et al., 1993; Clifton et al., 1998; Bakan et al., 2006), suggesting that defective insulin signalling in diabetes might be due to a defective membrane organisation (i.e. due to reduced receptor clustering in rafts). The numerous works showing changes in brain lipid composition with age (see Colin et al., 2016) could explain insulin signalling deficits in this organ as well. However interesting, these studies do not help to determine if lipidic changes determine pathology or they are (yet another) consequence of it.

### 3. Cholesterol in the brain

Cholesterol is an essential component of eukaryotic membranes. Its particular composition and characteristics make it essential in plasma membrane dynamics, especially relevant in the regulation of membrane fluidity, thickness and generation of sub-compartments (lipid rafts), with specific organisation and roles. The substantial role of cholesterol in the formation of these sub-domains is particularly pivotal, as these sub-compartments act as scaf-

folds for different and crucial signalling platforms, including insulin (Simons et al., 1997). Due to these singular characteristics, cholesterol has the capacity to influence ion channel permeability, death-survival signalling, exo-endocytosis, gene expression and, especially relevant for an organ like the brain, synaptic vesicle release and neurotransmitter receptor fusion and internalisation (Maguire et al., 1989; Valenza et al., 2005 and 2015; Lippincott-Schwartz et al., 2010; Mailman et al., 2011 Levitan et al., 2014). Therefore, a significant alteration in cholesterol levels and homeostasis in brain cells will undoubtedly have deleterious consequences. In fact, numerous works have linked brain cholesterol with cognitive defects in the normal old and with the occurrence of neurodegenerative features. Below, we will summarise the main features of cholesterol general homeostasis and functions in the brain, focusing then in its relationship with ageing and pathology.

### **3.1 Brain cholesterol**

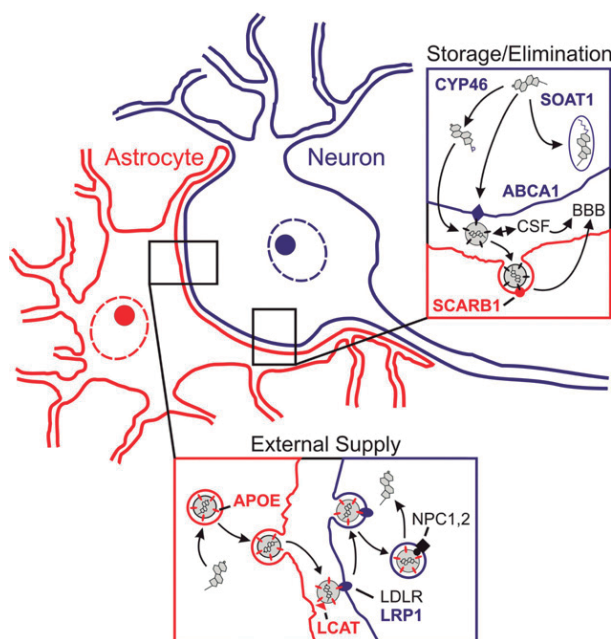
#### *3.1.1 General considerations:*

Although the brain represents 2% of body mass, it contains 23% of the whole cholesterol content. This clearly indicates the crucial importance of this lipid for brain organisation and function. The greatest proportion of brain cholesterol is present in the myelin sheaths, an essential component for the propagation of action potentials along axons. The rest of the cholesterol in the brain is present in the plasma membrane and endo-membranes of other brain cells: neurons, astrocytes and microglia. Yet, due to their size, architectural complexity and functional properties, it is thought that neurons are extremely sensitive to changes in cholesterol content. This is corroborated by the observation that the brain is the main affected organ in hereditary conditions of gene mutations in cholesterol synthesis/transport genes, despite their presence in all cells of the organism (reviewed in Martin et al., 2014a). Another peculiarity of brain cholesterol is that all cholesterol in the brain is the product of synthesis by brain cells, since the BBB blocks the entry of blood lipoprotein-cholesterol particles. Therefore, the levels of cholesterol in the brain are not to be related with peripheral alterations (Björkhem et al., 2004).

#### *3.1.2 Cholesterol synthesis:*

The first step in cholesterol synthesis in all the tissues consists in the transformation of acetyl-CoA in 3-hydroxy-3-methylglutaryl-CoA by HMG-CoA synthase (Konrad, 1992). The reduction of this product through HMG-CoA reductase (HMG-CoAR) results in Mevalonate, a molecule that will be subject to a 19-step process of enzymatic reactions to finally yield cholesterol (Berg et al., 2002). The initial steps in cholesterol synthesis take place in the endo-

plasmic reticulum (ER), nevertheless it is not abundant in this organelle, where it represents a 1% of total cell cholesterol (Ikonen et al., 2008). Once synthesised it is delivered mainly to the plasma membrane, where it accounts for a 25% of total lipids. Other membranes rich in cholesterol are the endocytic recycling compartment and the Golgi apparatus, mostly enriched in the *trans*-Golgi compartment. The cholesterol level in the cell is precisely tuned by the SREBPS system. The SREBPs (sterols regulatory element-binding proteins) are membrane-bound transcription factors settled in the ER. When these proteins sense a reduction in cholesterol they are delivered to the Golgi, where upon activation they translocate to the nucleus to regulate the transcription of cholesterol synthesis genes (HMG-CoAR) and lipoprotein (cholesterol transporter molecules) receptor genes (LDLR) (Smith et al., 1988 and 1990).



**Figure I6. Cholesterol production in glial cells and delivery to neurons.**

The diagram shows how astrocytes (red) produce and deliver cholesterol to neurons, using lipoproteins like APOE. Enzymes like LCAT, that esterified cholesterol allowing it to enter the lipoprotein, could modify the cholesterol present in these lipoproteins. The acquisition of lipoproteins by neurons (blue) is mediated by receptors like LDLR and LRP1. Once inside the neurons cholesterol is released from endosome/lysosome system by NPC proteins that allow it to go to its destinations. The excess of cholesterol could be stored esterified by enzymes like SOAT1, expelled to CSF in lipoproteins through transporters like ABCA1 or degraded to 24OHC by CYP46A1 enzyme. Adapted from Pfrieger et al., 2011.

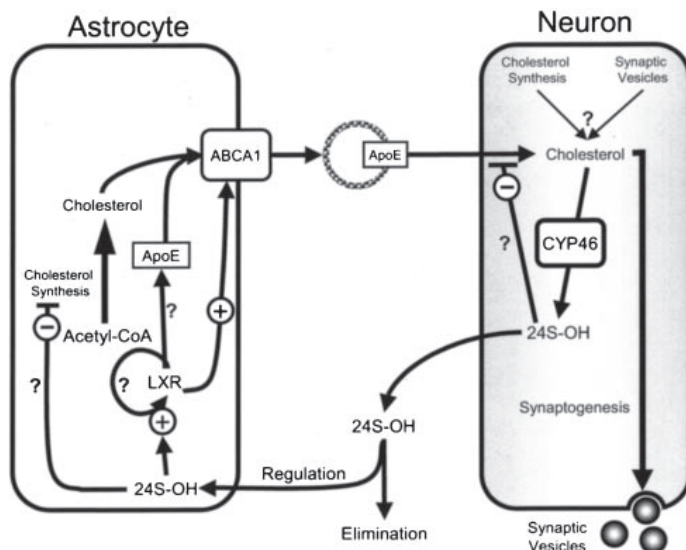
Cholesterol synthesis varies among the different cell types of the brain and also in relation to time of development. Cholesterol synthesis is at high rate during brain postnatal development and significantly decreases with age (Quan et al., 2003; Thelen et al., 2006). Regarding synthesis rates in the different brain cells, oligodendrocytes produce their own cholesterol as so do astrocytes at all times. Neurons on the other hand are self-dependent at initial stages of development but once myelination is completed, in early life, they rely on astrocytes synthesis and delivery (Fünfschilling et al., 2007). It has been postulated that the astrocyte dependence is a way to spare energy to neurons, due to the high amounts of energy and resources that cholesterol synthesis requires (Mauch et al., 2001). The pathway of astrocyte-to-neuron cholesterol provision is schematised in Figure I6. In short, synthesised

cholesterol by astrocytes is stored in the form of esterified cholesterol. Upon secretory signaling, astrocytes' lipoproteins, APOE (apolipoprotein E, one of the most important), APOJ/CLU or APOD form high-density lipoproteins (HDL) with esterified cholesterol (at lower levels than the HDL particles in serum) and several cholesterol precursors, as desmosterol or lathosterol, that then the neurons transform in cholesterol (DeMattos et al., 2001; Mutka et al., 2004). Cholesterol transfer to the particles is mediated by ATP binding cassette (ABC) transporters (Fukumoto et al., 2002; Tarr et al., 2008). These particles are secreted to the interstitial space by mechanisms similar to those employed by hepatocytes, i.e. assembly in the ER, transfer to the Golgi and secretion.

Uptake of cholesterol lipoparticles by neurons is mediated by specific receptors that bind APOE and the other lipoproteins. The most important receptors in neurons are LRP1 (mainly expressed in neurons) and LDLR (that can also be expressed in astrocytes) (Pfrieger et al., 2011). Upon internalisation by receptor-mediated endocytosis, cholesterol follows the endosomal/lysosomal system, where it is released and delivered back to the plasma membrane or to internal organelles. One mechanism of release involves the NPC2 protein present in the lumen of late endosomes. This protein binds unesterified cholesterol from lipoproteins and transfers it to the NPC1 protein. This is a transmembrane protein with a sterol sensitive domain that mediates the exit of cholesterol from the endosomal/lysosomal system (Storch et al., 2009). Additionally to this mechanism, cholesterol taken up from astrocytes might be stored in its esterified form. This comes from the observation that neurons express the ACAT1/SOAT1 proteins, which have been involved in cholesterol esterification, (Sakashita et al., 2000). In addition to cholesterol uptake mechanisms, neurons possess a well-developed system for removing it. Despite there being studies that describe expression of ABC transporters in neurons, and the possibility of direct cholesterol excretion on APOA1 or APOE-containing lipoproteins (Fukumoto et al. 2002), the most active pathway for cholesterol removal in neurons is its conversion to 24S-hydroxycholesterol (24OHC) by the enzyme cholesterol 24-hydroxylase or CYP46A1 (Figure I7).

CYP46A1 is mainly expressed in different neurons in the brain and little in astrocytes (Ramirez et al., 2008). The 24OHC product generated by its action is able to translocate through the different lipophilic compartments in the brain, including the BBB, being the main mechanism by which the brain eliminates the excess of cholesterol. In addition, 24OHC released from neurons plays a fundamental role in the control of brain cholesterol content by means of its binding to LXR receptors in the astrocytes. Binding triggers the expression of proteins like APOE or ABCA1 implicated in the excretion of cholesterol (Wang et al., 2008)

thus synchronising the cholesterol status of neurons and astrocyte to assure that this essential lipid is maintained at appropriate levels.



**Figure 17. 24OHC effects on cholesterol homeostasis.**

The diagram indicates how 24OHC, product of cholesterol degradation, is released to the CSF for its elimination, or could be incorporated by astrocytes. 24OHC activates LXR receptors in astrocytes stimulating cholesterol synthesis and delivery to neurons, thus controlling and regulating cholesterol homeostasis in the brain. Adapted from Björkhem et al., 2004.

### 3.2 Cholesterol in synaptic signalling

As previously mentioned, besides its important functions in membrane structural maintenance, cholesterol it is also implicated in the organisation of functional structures. Cholesterol is not homogeneously distributed in the membranes, appearing at higher levels in sub-domains known as lipid rafts that constitute by themselves functional entities with important relevance for cell signalling and trafficking (Simons et al., 1997). The formation of these specific structures allows the recruitment into signalling platforms of particular proteins that on the one hand participate in downstream events and at the same time modulate the proper organisation of the rafts. Although not exclusively, lipid rafts are abundant at and around synapses, where they have been found to contribute to neurotransmission and also to receptor-mediated intracellular signalling (Cooper et al., 1984; Pfrieger et al., 2003; Oh et al., 2007; Chen et al., 2008). It is in this regard where the control of brain cholesterol levels throughout life acquires maximal relevance, as any content change, in more or in less, will certainly influence synapse function and, as a consequence, brain performance.

#### 3.2.1 Cholesterol in presynaptic terminals:

The release of neurotransmitters from presynaptic terminals is a crucial step for the correct transmission of neuronal signals. The neurotransmitters are molecules produced by neurons for spreading their electrical signalling. They are accumulated in vesicles that remain close to the plasma membrane ready to fuse with it under a signal and release their contain

to the intracellular space. In this releasing process the protein complex SNARE is crucial for accomplishing the fusion of the vesicles to the plasma membrane (Sollner et al., 1993; Takizawa et al., 1993). This process requires a perfect coordination between the different components of the plasma membrane, lipids and proteins. In this complex organisation, cholesterol plays an important role and it is revealed as an essential element of the exocytosis structure. Different studies revealed the presence of high levels of cholesterol in presynaptic terminals and it was shown that the reduction of cholesterol causes a decrease in vesicle exocytosis (Mailman et al., 2011). The intrinsic negative curvature of cholesterol, a property required for vesicle merging process, could explain this situation (Jahn et al., 1999). It has also been described an association between SNARE complexes and lipid rafts (Lang et al., 2001). Thus the alteration of cholesterol levels could lead to destabilisation of exocytosis protein complexes, affecting the capacity of the neurons to trigger the neurotransmitters release (Chamberlain et al., 2001). Likewise, different publications linked cholesterol with vesicle formation in neurons, reporting a relation between cholesterol levels and rate of vesicle generation (Hannah et al., 1999). Finally, other studies revealed that kinesin-mediated vesicle transport in the cell requires the presence of lipid rafts (Klopfenstein et al., 2002), which could also relate cholesterol with neurotransmitters delivery.

### 3.2.2 Cholesterol in postsynaptic terminals:

Presynaptic structures find their counterparts in the so-called postsynaptic terminals. These regions contain the receptors to the different neurotransmitters that are produced and released by neurons, as well as other kind of receptors and ion channels also related with synaptic transmission. The particular organisation of the plasma membrane in lipid rafts, make them suitable to cluster and potentiate the activity of all these types of receptors and channels. This however does not imply that all receptors and channels are present in lipid rafts, but instead that lipid rafts serve as transient platforms facilitating receptor organisation, lateral diffusion and gathering in functional complexes finally favouring signal transference. In fact, some receptors and channels have been found in lipid rafts, as for example nicotinic acetylcholine receptors (AChRs), AMPA receptors, GABA receptors and also scaffolding proteins that form part of these postsynaptic structures like PSD95 protein or glutamate-receptor interacting protein (GRIP) (Brückner et al., 1999; Brusés et al., 2001; Suzuki et al., 2001).

Again, as one of the main components of these lipid rafts, a change in cholesterol levels in neurons leads to alterations in postsynaptic terminals. This is exemplified in the increase in glutamate receptor-mediated currents when cholesterol levels are increased (Mauch et al., 2001). Another example is the increasing accumulation of AMPA receptors with cholesterol

reduction, due to their impaired lateral mobility and decreased endocytosis (Martin et al., 2014b). In addition to these direct effects in the regulation of pre and postsynaptic currents, cholesterol plays a role in the stability of the synapse through affecting the properties of adhesion proteins present in synaptic and peri-synaptic lipid rafts (He et al., 2002; Leitinger et al., 2002). Thus, neuronal membrane cholesterol controls critical aspects of synaptic transmission, at the level of channels and receptors but also at the architectural level.

### **3.3 Cholesterol in Alzheimer's disease and ageing**

Cholesterol homeostasis in brain cells has been revealed a critical issue during lifespan. As previously shown it has great importance for the correct maintenance of membranes structure and particularly in neurons is intimately linked with correct synaptic performance. Thereby it is easy to assume that cholesterol homeostasis deregulation in the CNS could cause or at least influence the cognitive process. In fact, there are numerous works showing that defects in brain cholesterol homeostasis play a critical role in the cognitive deficits of the old, but also in the development of neurodegenerative diseases, like Alzheimer's disease (reviewed in Martín et al., 2014a).

#### **3.3.1 Alzheimer's Disease:**

A relationship between cholesterol with AD has been suggested by different types of data, including human retrospective studies, genetic association and direct biochemical experimentation in laboratory animals (reviewed in Martin et al., 2014a). Hypercholesterolemia is a major risk for AD, though the cause for it may relate to brain hypo-perfusion, inflammation, endothelial cell dysfunctions or blood-brain barrier breakdown rather than a reflection in the changes in cholesterol content (Altman et al., 2010; Vasilevko et al., 2010). As mentioned, high levels of blood cholesterol are related with insulin resistance, in turn affecting amyloid processing, among other alterations (Kruit et al., 2010; Matsuzaki et al., 2010). Another mechanism by which high circulating cholesterol may favour AD is by the excess of cholesterol catabolic products, the oxysterols. Circulating oxysterols worsen the atherogenic process and induce vascular cells apoptosis (Poli et al., 2009). Yet the most conclusive data linking high cholesterol with AD is the inheritance of the  $\epsilon 4$  allele of the *ApoE* gene. The presence of this allele is related to cardiovascular diseases and atherogenicity (Schmechel et al., 1993), and at the brain level with impaired cholesterol transport, reduced myelin formation and lower capacity to degrade beta amyloid peptide (Martin et al., 2014a). In light of the numerous observations that cholesterol levels are diminished in AD brains (see reviews by Martin et al., 2014a and by Colin et al., 2016) it appears reasonable to propose that brain cholesterol metabolism is a sensitive indicator of body homeostasis. The mechanisms by which the brain

reduces cholesterol content in the hypercholesterolemic background are not known, though they might have different origins; among others, impaired cholesterol uptake by neuronal cells (Beel et al., 2010), inhibition of cholesterol synthesis by amyloid beta peptide, modification in lipid rafts (Evangelisti et al., 2014) or increased CYP46A1 activity leading to higher levels of degradation products and lower levels of cholesterol (Lütjohann et al., 2000). Hence, the association between high blood cholesterol with AD appears to be the consequence of a brain vascular response to hypercholesterolemia resulting, among other numerous consequences, in changes in brain cholesterol homeostasis, with its obvious functional alterations.

### 3.3.2 Ageing:

It has been previously reported that brain lipidic composition changes with age. In particular different groups have reported a cholesterol reduction, both in the human brain and in rodents (Svennerholm et al., 1994 and 1997; Martin et al., 2014a, Colin et al., 2016), being the hippocampus one of the regions affected. The mechanisms proposed to explain this loss are multiple: age-reduced synthesis, age-reduced astrocyte-neuron communication and age-increased levels of the cholesterol hydroxylating enzyme CYP46A1 (see Martin et al., 2014a). Changes in this enzyme seem to be particularly relevant for the age associated cholesterol deficit: i) its levels are regulated at the transcriptional level by oxidative metabolites (Ohyama et al., 2006), a typical feature of aged cells, and ii) oxidative stress increases the enzyme's mobilisation from internal reservoirs (the ER) to the plasma membrane (Sodero et al., 2012). Expectedly, age-associated cholesterol loss was correlated with several synaptic plasticity deficits of the old. Thus, it was reported that cholesterol loss impairs long-term depression (LTD) through altering NMDA receptors signalling, leading to impaired activation of the CaMKII-CREB/CBP and, by these means, decreased histone deacetylase 4 (HDAC4) displacement from repressed chromatin resulting in reduced transcription of early memory genes (Palomer et al., 2016a). It was also observed, that age-associated cholesterol loss is behind the upregulation of TrkB activation, in turn leading to upregulation of Akt signalling and impaired AMPA receptors mobilisation and internalisation, both events required for LTD induction (Martin et al., 2014b). Furthermore, cholesterol loss was also shown to affect long-term potentiation (LTP) by a mechanism that involves the detachment from plasma membrane of the protein MARKCS. The lower MARKCS levels in turn induce a reduction in PI(4,5)P<sub>2</sub> levels leading to decrease PLC $\gamma$  activity (Trovò et al., 2013). All in all, these data shed light into some of the mechanisms by which changes in cholesterol homeostasis with age could be partly responsible for the deficits in cognition that characterise this stage of life.

# *OBJECTIVES*

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The insulin and IGF-1 signalling's functions and roles in the brain have been gaining interest lately due to their increasing importance in learning and memory events. Despite the numerous works showing the existence of insulin and IGF-1 signalling deficits with age and in pathological conditions like Alzheimer's, and that these deficits are associated with cognitive decay, it is still missing a direct demonstration that age affects insulin/IGF-1 synaptic plasticity functions. Hence, the objectives we have tackled during this thesis were:

1. To determine the effect of age on insulin-mediated synaptic plasticity.
2. To determine the effect of age on the molecular mechanisms responsible for insulin-mediated synaptic plasticity.
3. To attempt to improve insulin/IGF-1 signalling in the old.

## *MATERIALS & METHODS*

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### **1. Human embryonic kidney (HEK) cells in culture**

Hek 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM Glutamine, 100 IU/ml Penicillin and 100 mg/ml Streptomycin (complete DMEM). Cells were incubated at 37°C, humidity conditions and 5% CO<sub>2</sub>.

### **2. Primary hippocampal neurons**

Hippocampal neurons were extracted from Wistar rat embryos at embryonic day 18 (E18) and seeded in culture conditions as previously described (Kaeck et al. 2006). Briefly hippocampi were dissected into ice-cold Hank's solution (Hank's buffer salt solution (HBSS) Ca<sup>2+</sup> and Mg<sup>2+</sup> free, Gibco, Life Technologies Co.) with 7 mM HEPES and 0.45% glucose. We treated the hippocampi with 0.005% trypsin (Trypsin-EDTA 0.05%, Gibco, Life Technologies Co.) and kept them at 37°C for 16 min. Tissue was washed three times with Hank's solution and then dissociated in 5 ml of plating medium (Minimum Essential Medium (MEM) supplemented with 10% horse serum and 20% glucose). Cells were counted using a Neubauer Chamber and plated into poly D-lysine 0.1% (Sigma-Aldrich) pre-coated dishes (250000 cells in a 6 cm culture dish).

Plating medium was replaced after 4 hours by neurobasal media supplemented with B27 and GlutaMAX (Gibco, Life Technologies Co.). After 7 days *in vitro* (DIV) medium was replaced by the same medium without GlutaMAX. We maintained the neurons in culture at 37°C, humidity conditions and 5%CO<sub>2</sub>, until they were fully mature at 15 DIV.

### **3. Mouse hippocampal slices**

Hippocampal slices were obtained from C57BL/6J mice, using 7-12 month-old mice as adult mice and 20-24 month-old mice as old mice. Hippocampi were extracted and placed in Dissection solution (10 mM D-glucose, 4 mM KCl, 26 mM NaHCO<sub>3</sub>, 233.7 mM sucrose, 5 mM MgCl<sub>2</sub>, 1:1000 Phenol red) oxygen saturated with carbogen (95% O<sub>2</sub> / 5% CO<sub>2</sub>), and sliced using an automatic tissue chopper (McIlwain Tissue Chopper, Standard Table, 220 V, Ted Pella Inc.) to obtain 400 µm hippocampal slices. Then slices were kept in artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 11 mM glucose, 1.2 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, osmolarity adjusted to 290 Osm) oxygen saturated with carbogen for 1 hour. Experiments were performed in ACSF oxygen saturated.

#### **4. Drug treatments**

Different drugs and compounds were prepared for hippocampal neurons and Hek 293T cells treatment. Stock solutions of insulin-like growth factor-1 receptor  $\beta$  (IGF-1R $\beta$ ) and insulin receptor  $\beta$  (IR $\beta$ ) inhibitor Osi-906 (50 mM Selleckchem ref.: S1091), IGF-1R $\beta$  inhibitor PPP (10 mM Calbiochem ref.: 407247), p38 inhibitor PH797804 (10 mM Selleckchem ref.: S2726) and Jnk inhibitor SP600125 (50 mM Tocris ref.: 1496) were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Stock solutions of Cholesterol oxidase (Choox, 500 IU/ml, Calbiochem ref.: 228250) and human peptide purified IGF-1 (13  $\mu$ M, Stem Cell Technologies #78022) were prepared in Milli-Q water.

Hippocampal neurons were treated with 1  $\mu$ M Osi-906 (1 hour), 1  $\mu$ M PPP (1 hour), 2  $\mu$ M PH797804 (1 hour) and 10  $\mu$ M SP600125 (1 hour) prior to cholesterol removal with 10 IU/ml Choox (30 min). Hek 293T cells were treated with 4  $\mu$ M IGF-1 (25 min) and 40 IU/ml Choox (1h) before paraformaldehyde (PFA) fixation for FRET experiments (see below paragraph 14). All treatments on hippocampal neurons or Hek 293T cells were performed at 37°C.

Experiments for cholesterol addition conducted in hippocampal slices were performed at 25°C. Methyl- $\beta$ -cyclodextrin-cholesterol (M $\beta$ CD-Ch) solution was prepared freshly at use concentration in ACSF, containing 30  $\mu$ M Cholesterol Water-soluble (Sigma-Aldrich ref.: C4951) and 5  $\mu$ M Cholesterol (Sigma-Aldrich ref.: C3045). After hippocampal slices preparation and recovery (1 hour in ACSF oxygen saturated), hippocampal slices were treated with M $\beta$ CD-Ch solution (1 hour) for cholesterol addition and collected for further processing.

#### **5. Animal handling**

Male C57BL/6J mice were used in this study: adult 7-12 month-old and old 20-24 month-old. All the animals were kept in the Centro de Biología Molecular Severo Ochoa's (CBMSO) animal facility, with food and water available *ad libitum*. Animals were maintained in a temperature-controlled environment, following a 12-12 light-dark cycle beginning light at 7 a.m. All the experiments were performed in accordance with European Union guidelines (2010/63/UE) regarding the use of laboratory animals.

## 6. *Animal treatment*

Voriconazole (from HangZhou Dayangchem Co., CAS No: 137234-62-9) was solubilised using hydroxypropyl- $\beta$ -cyclodextrin (Sigma-Aldrich ref.: H107): 15 g of hydroxypropyl- $\beta$ -cyclodextrin were dissolved in 100 ml of saline solution (0.9% NaCl) and heated to 80°C in a water bath with stirring. Then 1.5 g of Voriconazole were added to the cyclodextrin solution with stirring at 80°C until complete dissolution. This stock solution (15 mg Voriconazole/ml cyclodextrin/saline) was conserved at 4°C protected from light. On the first day of experimentation, an aliquot of this solution was diluted in drinking water to a final concentration of 0.68 mg/ml Voriconazole. Considering that a mouse drinks 3 ml water per day, the dose corresponds to 2.04 mg/day. The average weight of a mouse is 34 g, resulting therefore in a dose 60 mg/kg body weight. Water with this concentration of Voriconazole was used as the hydration source in 20 month-old mice during 45 days. Vehicle 20 month-old mice received the same amount of hydroxypropyl- $\beta$ -cyclodextrin than Voriconazole mice during 45 days. Bottles containing the Voriconazole or Vehicle water were changed weekly.

## 7. *Protein extracts processing*

Protein extracts were obtained from cultured hippocampal neurons or hippocampi extracted from C57BL/6J mice and homogenised in RIPA buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail 2 Sigma-Aldrich and protease inhibitor Complete Roche). Extracts for cholesterol measurements either from hippocampal neurons or from mice hippocampi were homogenised in MES buffer (25 mM 2-N-Morpholino ethanesulfonic salt pH7, 2 mM EDTA, phosphatase inhibitor cocktail 2 and protease inhibitor Complete). Protein concentration was determined using the colorimetric assay BCA (Pierce ® BCA protein assay kit, Thermo Fisher Scientific)

## 8. *Electrophoresis and protein detection by Western Blot*

Protein extracts were boiled at 99°C for 5 min. in Laemmli Buffer (125mM Tris pH 6.8, 1% SDS, 3.48% glycerol, 0.4%  $\beta$ -Mercaptoethanol and 1.2  $10^{-4}$ % Bromophenol blue). Then samples were electrophoretically resolved within reduced and denaturing polyacrylamide gels (8% or 10% polyacrylamide) and afterwards transferred to nitrocellulose membranes using Mini-Protean system (Bio-Rad) 380mA 1 hour and 45 min. Membranes were blocked for 1 hour in PBST (1X PBS – 0.1% Tween-20 (v/v)), containing 5% BSA (Sigma-Aldrich), and incubated overnight at 4°C with the antibodies (Table 1) diluted in PBST - 5% BSA. Peroxidase-conjugated polyclonal goat anti-rabbit (Dako ref.: P0448), peroxidase-conjugated polyclonal rabbit anti-mouse (Dako ref.: P0260), fluorophore-conjugated polyclonal donkey

anti-mouse (Li-Cor ref.: 926-68022) and fluorophore-conjugated polyclonal goat anti-rabbit (Li-Cor ref.: 926-32211) were used as secondary antibodies (at 1:5000 the peroxidase-conjugated and 1:2500 the fluorophore-conjugated) for 1 hour at room temperature in PBST - 5% BSA. Bands were visualised with Super Signal™ chemiluminescent substrate (Thermo Fisher Scientific) in an ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences), or with an Odyssey Infrared imaging system (B446-Li-Cor) in the case of the fluorophore-conjugated antibodies. Bands were quantified with ImageJ.

## **9. Cholesterol quantification**

Neuronal and hippocampi extracts were homogenised as previously described in MES buffer (see paragraph 7). Protein concentration was quantified using BCA assay kit. Then cholesterol quantification was determined per  $\mu\text{g}$  of protein using the Amplex® Red Cholesterol Assay Kit (Invitrogen). Amplex Red kit allows cholesterol levels detection through transformation of cholesterol molecules into 4-Cholesten-3-one plus  $\text{H}_2\text{O}_2$  by means of *Cholesterol oxidase* activity.  $\text{H}_2\text{O}_2$  generated is used by *Horseradish peroxidase* (HRP) enzyme for oxidizing the Amplex Red compound, producing as a result *Resorufin*, a product that emits high fluorescence levels. After incubating samples with the Amplex Red solution (Amplex Red, HRP, Reaction buffer and *Cholesterol oxidase*), the fluorescence emitted was measured with a FluoStar OPTIMA (BMG labtech) fluorometer using 560 nm as excitation and 590 nm as emission wavelength.

## **10. Immunoprecipitation**

Protein concentration was quantified in hippocampal neuron extracts as previously described (see paragraph 7). Samples were pre-cleared using 25  $\mu\text{l}$  protein A/G agarose beads (Santa Cruz ref.: SC-2003) and 10  $\mu\text{g}$  of normal IgG (Santa Cruz ref.: SC-2025 for normal mouse IgG and SC-2027 for normal Rabbit IgG) for each 150  $\mu\text{g}$  of protein. Samples were placed in a rotor for 1-3 hours at 4°C. Then samples were centrifuged and the supernatant collected. Afterwards the antibodies (Table 1) were added to the supernatant together with 25  $\mu\text{l}$  protein A/G agarose beads and the mixture was incubated in a rotor overnight at 4°C. As a negative control non-immune normal IgGs were used in place of specific antibodies. Immunoprecipitated complexes were washed four times with 1X PBS buffer plus 0.1% NP-40 (Sigma-Aldrich). Complexes were eluted with Laemmli Buffer for 5 min. at 99°C and proteins were identified by Western Blot.

## 11. Antibodies

Primary antibodies used for protein detection by Western Blot and Immunoprecipitation.

**Table 1**

<i>Antibody</i>	<i>Isotype</i>	<i>Origin</i>	<i>Reference</i>	<i>Dilution WB</i>	<i>Dilution IP</i>
$\alpha$ -Tubulin	Mo	Abcam	ab7291	1:10000	
$\beta$ -Actin	Mo	Sigma-Aldrich	A5441	1:20000	
Gapdh	Mo	Abcam	ab8245	1:20000	
Akt	Rb	Cell Signaling	#9272	1:1000	
P-Akt (Ser473)	Rb	Cell Signaling	#4060	1:1000	
Gsk3 $\alpha/\beta$	Mo	Invitrogen	44-610	1:1000	
P-Gsk3 $\alpha/\beta$ (Ser9)	Rb	Cell Signaling	#9331	1:1000	
IGF-1 Receptor $\beta$	Rb	Cell Signaling	#9750	1:1000	1:100
P-IGF-1 Receptor $\beta$ (Tyr1135/116)	Rb	Cell Signaling	#3024	1:1000	
Insulin Receptor $\beta$	Mo	Santa Cruz	SC-57342		1:100
Insulin Receptor $\beta$	Rb	Santa Cruz	SC-711	1:750	
P-Insulin Receptor $\beta$ (Tyr1150/1151)	Mo	Millipore	04-299	1:750	
IRS-1	Rb	Millipore	05-784	1:1000	
IRS-1	Mo	BD Transduction Laboratories	611394	1:1000	
P-IRS-1 (Ser312)	Rb	Abcam	ab4865	1:750	
P-IRS-1 (Ser636/639)	Rb	Cell Signaling	#2388	1:1000; 1:200	
P-IRS-1 (Ser636)	Rb	Santa Cruz	SC-101711	1:500	
SAPK/Jnk	Rb	Cell Signaling	#9252	1:1000	
P-SAPK/Jnk (Thr183/Tyr185)	Rb	Cell Signaling	#9251	1:1000	
p70S6 Kinase	Mo	BD Transduction Laboratories	611260	1:1000	
P-p70S6 Kinase (Thr389)	Mo	Cell Signaling	#9206	1:750	
Vinculin	Rb	Millipore	AB6039	1:1000	
p38 $\alpha$	Rb	Cell Signaling	#9218	1:750	
p38 $\alpha$	Rb	Abcam	ab170099	1:1000	
P-p38 (Thr180/Tyr182)	Rb	Cell Signaling	#4511	1:750	

## **12. Insulin Receptor $\beta$ sensitivity experiment**

Insulin Receptor  $\beta$  sensitivity was determined in mouse hippocampus by measuring the capability of the receptor to become active upon ligand supply. For that purpose adult and old male mice were intraperitoneally injected with 27 IU/Kg body weight of human purified insulin (Actrapid®, Novo Nordisk) diluted in saline solution (0.9% NaCl). Injected mice were kept 1 hour at regular conditions (see paragraph 5) and then sacrificed. Hippocampi were extracted and processed as previously described. Samples were immunoprecipitated using an antibody against insulin receptor  $\beta$  and the level of receptor phosphorylation determined by Western Blot.

## **13. Insulin Tolerance Test**

Adult and old male mice were fasted for 4 hours in clean cages without any food. Glucose concentration was determined in blood samples (from the tail vein) at the end of fasting (0 time-point) using an automatic analyser (Accu-Chek Aviva, Roche). Then fasted animals were intraperitoneally injected with 1IU/Kg body weight of human purified insulin (Actrapid®, Novo Nordisk) in saline solution (0.9% NaCl). Glucose concentration was determined at times 15, 30, 45 and 60 min. after insulin injection.

## **14. Insulin-like growth factor-1 Receptor $\beta$ activity**

Insulin-like growth factor-1 receptor  $\beta$  activity was measured by fluorescence resonance energy transfer (FRET) in Hek 293T cells transfected with insulin-like growth factor-1 receptor  $\beta$  extracellular and transmembrane regions fused to EYFP (FRET donor) or mCherry (FRET acceptor), in the presence of IGF-1 or cholesterol oxidase (Choox). Plasmids were provided by Dr. Patrick. O. Byrne and Dr. Daniel J. Leahy from Johns Hopkins University School of Medicine, Baltimore, US (Kavran et al., 2014).

### **14.1 Plasmid expression:**

Plasmids were expressed using 2  $\mu$ l polyethylenimine (PEI) reagent (Polysciences ref.: #23966-2) for each 0.5  $\mu$ g plasmid and 250  $\mu$ l OPTIMEM (Gibco, Life Technologies Co.). Plasmid and PEI reagent were mixed in OPTI-MEM and incubated at room temperature for 30 min. Then the mix was added to Hek 293T cells maintained in complete DMEM in a 1:5 (v/v) proportion. Cells were incubated with transfection mix for 4 hours at 37°C. Afterwards medium with 1:5 of transfection mix proportion was replaced by complete DMEM and cells were incubated at regular conditions. Expression of the transfected plasmid was detectable 16 hours after transfection.

### 14.2 Sample preparation:

24 hours after transfection, cells were harvested and plated at 80% confluence into 3.5 cm culture dish with 15 mm diameter coverslips previously coated with 1% poly D-Lysine. 24 hours later cells were washed three times in 1X PBS to remove all traces of FBS and then serum starved in DMEM without FBS and Glutamine. After 5 hours of starvation 4μM IGF-1 or 40 IU/ml Choox were added for 25 min. or 1 hour, respectively. Then the medium was removed and cells were washed once with 1X PBS, followed by 1% PFA fixation for 15 min. at room temperature. Finally PFA was removed and cells were washed four times in 1X PBS and mounted onto slides using Mowiol-Dabco (Mowiol, Chabiochem) without antifading.

### 14.3 FRET (Fluorescence Resonance Energy Transfer):

A confocal LSM710 microscope (Zeiss) coupled to an inverted AxioObserver Z1 microscope (Zeiss) was used for conducting acceptor photobleaching FRET experiments. Images were acquired using the following wavelengths:  $\lambda_{exc}=488\text{nm}$ ,  $\lambda_{em}=503-532$  for EYFP;  $\lambda_{exc}=561\text{nm}$ ,  $\lambda_{em}=600\text{nm}-699\text{nm}$  for mCherry. Three pre-bleaching and four post-bleaching images were taken in both channels. Photobleaching was performed in delimited regions of interest (ROI) of the cells, in particular in the plasma membrane of the cells where the IGF-1Rβ is mainly located under physiological conditions. Images were taking using a 63X oil objective and the ROIs used were squares of regular size. Acceptor signal was bleached up to a 5% of its initial magnitude applying the highest laser intensity during thirty iterations to the selected ROI. Zeiss imaging software was used to calculate FRET efficiency using the following equation:

$$FRET \text{ Efficiency (\%)} = [(Donor_{post} - Donor_{pre}) / Donor_{pre}] \times 100$$

where  $Donor_{pre}$  corresponds EYFP signal before bleaching and  $Donor_{post}$  corresponds to final EYFP signal after bleaching. All images were background-subtracted. Different conditions were used in order to determine the presence of FRET upon cholesterol removal from the plasma membrane: Control situation, where cells were incubated only with starving medium; positive control situation where starving cells were incubated with IGF-1 peptide, the specific ligand for the protein that was being studied; study situation where starving cells were incubated with Choox in order to determine if this stimulus was able to induce FRET and subsequently approximation of the IGF-1Rβ homodimers. Three independent experiments for each of the three conditions were performed. Forty images in each condition and each experiment were subjected to acceptor-photobleaching technique and used to determine de FRET efficiency of IGF-1Rβ upon Choox treatment.

### **15. Electrophysiology, Insulin-LTD**

Adult and old mice (see paragraph 5) were tested for synaptic plasticity in the hippocampal CA1-region *in vitro*. Animals were killed by cervical dislocation and the hippocampus was rapidly dissected out into ice-cold (4°C) ACSF saturated with carbogen. ACSF for electrophysiology experiments consisted of: 124 mM NaCl, 4.9 mM KCl, 24.6 mM NaHCO<sub>3</sub>, 1.20 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM glucose, pH 7.4. Transverse slices (400 µm thick) were prepared from the dorsal area of the right hippocampus with a tissue chopper and placed into a submerged-type chamber, where they were kept at 32°C and continuously perfused with ACSF at a flow-rate of 2.2 ml/min. After 90 min. incubation, one slice was arbitrarily selected and a tungsten electrode was placed in CA1 stratum radiatum. Field excitatory postsynaptic potentials (fEPSPs) were recorded by placing a glass electrode (filled with ACSF, 3–7MΩ) in the stratum radiatum opposite the stimulating electrode. The time course of the field EPSP was measured as the descending slope function for all sets of experiments. After input/output curves had been established, the stimulation strength was adjusted to elicit a fEPSP-slope of 35% of the maximum and kept constant throughout the experiment. During baseline recording, 3 single stimuli (0.1 ms pulse width; 10 s interval) were measured every 5 min. and averaged for the 60 min. fEPSP values. Unless otherwise stated, insulin-mediated LTD was achieved by application of 500 nM insulin dissolved in ACSF for 30 minutes (Van Der Heide et al., 2005, Huang et al., 2004). During peptidergic induction, evoked responses were monitored 1, 4, 7 and 10 min. and then continually every 5 min. as above. Insulin (500 nM) was applied for 30 min., commencing at time zero; drug or test substances were applied 30 min. prior to until 30 min. after the start of insulin application (zero point).

Insulin was purchased as a desiccated powder and reconstituted according the manufacturers' instructions (Selleck Chemicals). The following compounds were used: Quercetin (Sigma-Aldrich ref.: Q4951) tested at 20 µM dissolved in ACSF; Wortmannin (Tocris ref.: 1232) used at 0.5 µM; SB203580 (Axon Medchem ref.: 1363) dissolved in 50% ethanol and used at 0.5 µM; PH797804 (Selleck Chemicals ref.: S2726) dissolved in DMSO and used 1µM; SP600125 (Tocris ref.: 1496) dissolved in DMSO and used at 1µM and Voriconazole (HangZhou Dayangchem Co.) tested at 10nM. The final concentration of the vehicle used either ethanol or DMSO was to 0.02% v/v in ACSF. In several studies, the vehicle alone was tested to establish that this did not affect Insulin-LTD.

**16. Statistical analysis**

Statistical analyses were performed with Graphpad Prism 5 (Graphpad Software Inc.). All values are presented as mean  $\pm$  SEM (standard error of the mean). Data normality and variances were tested by Shapiro-Wilk test. Mann-Whitney U-test, Kruskal-Wallis or Friedman test, with Dunn's adjustment for multiple comparisons, were used for nonparametric data. Student's t-test or ANOVA with Bonferroni's adjustment for multiple comparisons were used for parametric data. Asterisks in the figures indicate P values as follows: \* $<0.05$ ; \*\* $<0.01$ ; \*\*\* $<0.001$ .

# *RESULTS*

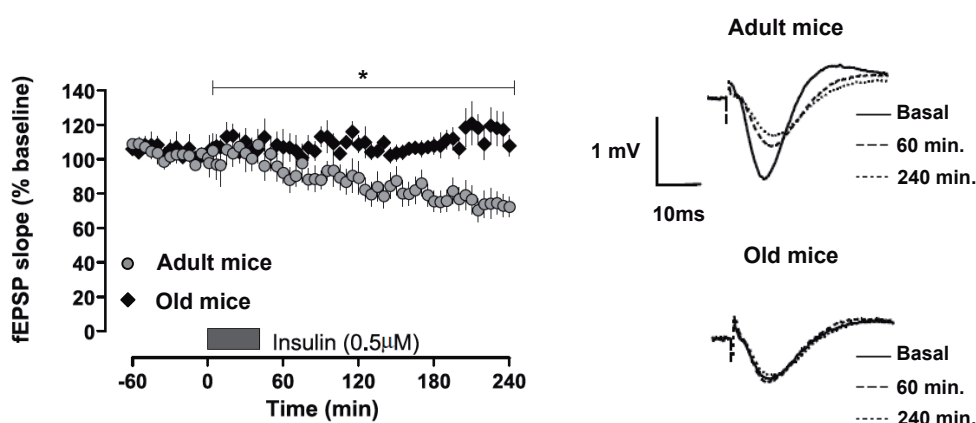
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## 1. Alterations in insulin signalling in old mice

### 1.1 Insulin-LTD is impaired in old mice:

Previous findings have revealed that the application of insulin to the hippocampus of young mice and rats leads to a significant depression of excitatory activity. This process is called insulin-dependent long-term depression or Insulin-LTD (Beattie et al., 2000; Lin et al., 2000; Man et al., 2000; Huang et al., 2003).

To determine the effect of age on this synaptic plasticity event, hippocampal slices from adult (7-12 month-old) and old mice (20-24 month-old) were incubated in the presence of 0.5  $\mu$ M insulin during 30 minutes. Analysis of the electrical response during the following 4 hours revealed significant age differences: insulin application caused a 20% depression of transmission at 3 hours after insulin addition, reaching a maximum of 30% reduction after 4 hours, in slices from adult mice but not in slices from old mice, which did not show any depression (Fig. R1). This result indicates that age impairs Insulin-LTD.



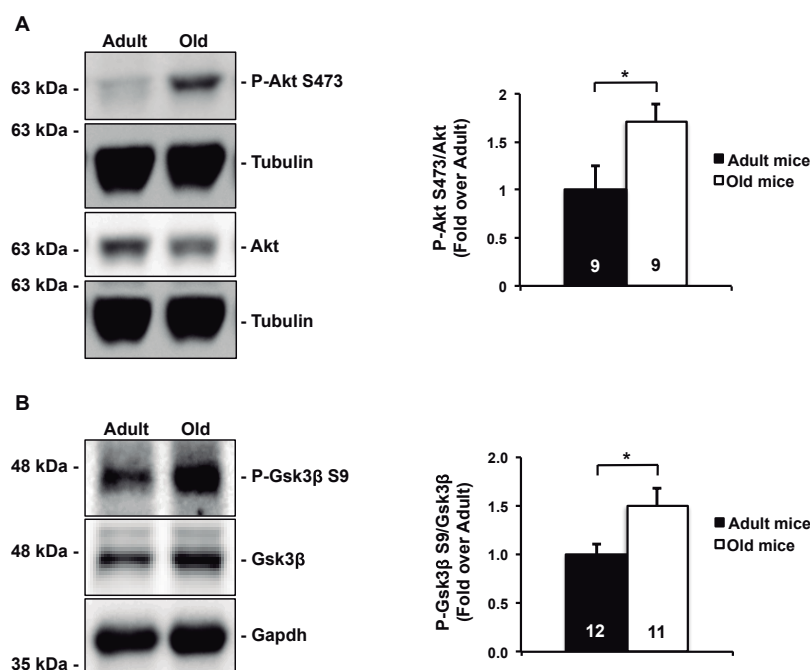
**Figure R1. Insulin-LTD is impaired in old mice hippocampus.**

Graph showing Insulin-LTD induction in adult mice (7-12 month-old;  $\circ$  grey filled;  $n=10$ ) and old mice slices (20-24 month-old;  $\blacklozenge$ ;  $n=7$ ). Insulin 0.5  $\mu$ M was perfused in ACSF for 30 min. The grey box indicates the time of insulin perfusion. The kinetic curves represent the field excitatory postsynaptic potentials (fEPSPs) as % baseline. The sample traces on the right represent the electric signal at different time points: Basal, 60 min. and 240 min. Data are represented as mean  $\pm$  SEM (One-way Anova;  $*p<0.05$ ).

### 1.2 Insulin signalling upregulation leads to pathway desensitisation in old mice:

In order to determine the mechanism underlying reduced Insulin-LTD in the hippocampus of old mice, we investigated the state of activity of insulin signalling (post-receptor) intermediaries. It is well known that activation of the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) leads to the phosphorylation of PI3K (phosphatidylinositol 3-kinase). PI3K then phosphorylates lipids on the plasma membrane, forming second messen-

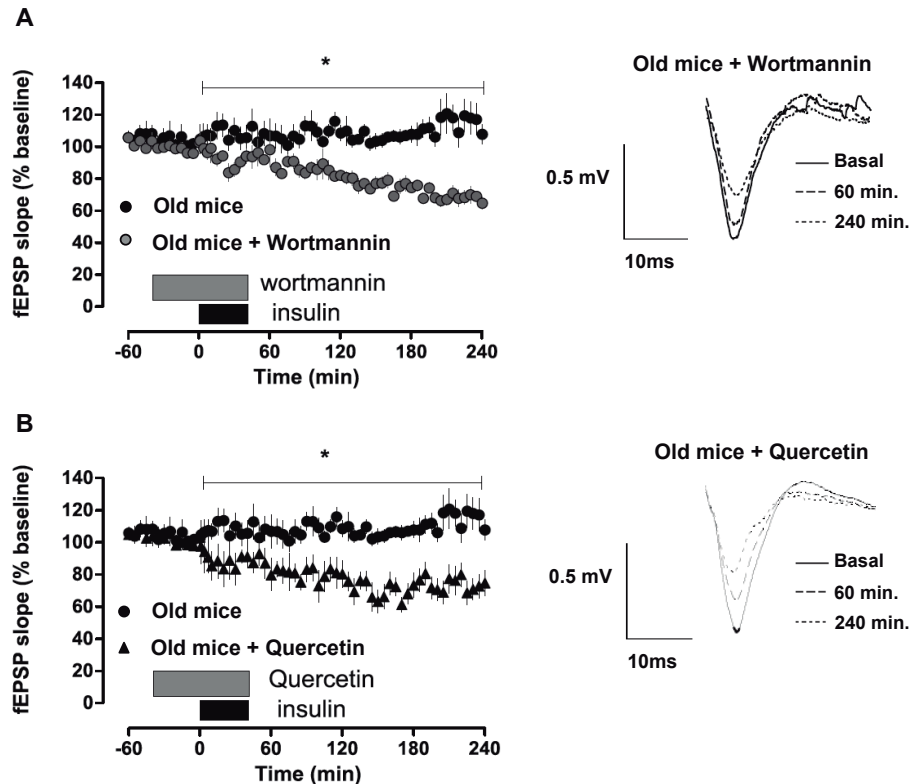
ger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Akt, a serine/threonine kinase, is recruited to the membrane by interaction with these phosphoinositide docking sites, so that it can be fully activated. Once activated by insulin, Akt mediates a number of downstream responses, including protein-synthesis mediated internalisation of AMPA receptors and LTD (Huang et al., 2004). Thus, the absence of Insulin-LTD in old mice hippocampi made us envision that the PI3K/Akt pathway could be inhibited in the hippocampus of old mice. Quite the contrary, as we observed that Akt was strongly activated in old mice respect to adult mice (Fig. R2A).



**Figure R2. High levels of active Akt and inhibited Gsk3β in old mice hippocampus.**

(A) Detection by Western Blot (WB) of active Akt levels using an antibody against the Akt activating mark Phospho-Serine 473. The levels of active Akt were measured in hippocampal extracts of adult (7-12 month-old) and old (20-24 month-old) mice. (B) Levels of the Gsk3β inhibiting mark Phospho-Serine 9 detected by WB in hippocampal extracts of adult and old mice. Data are represented as mean ± SEM (t-test; \*p<0.05).

To confirm Akt activation in these samples, we checked the levels of a known downstream Akt target, the Serine 9 inhibiting mark in Gsk3β. In agreement with the strong Akt activity, Gsk3β showed higher levels of its inhibitory mark in old mice (Fig. R2B). In order to test if the high PI3K/Akt pathway activity plays a role in the Insulin-LTD impairment observed in old mice, we inhibited PI3K/Akt signalling during stimulation of hippocampal slices with insulin, following the same protocol as before (see Fig. R1). Both Wortmannin, a canonical PI3K inhibitor, and the naturally found flavonoid Quercetin, which efficiently inhibits Akt (Gulati et al. 2006), were capable of recovering the sensitivity to exogenous insulin in hippocampal slices from old mice (Fig. R3A-B). The lack of insulin function in the old hippocampus in spite of high downstream (PI3K/Akt) activity could be explained by the occurrence of insulin pathway resistance due to pathway desensitisation, leading to the failure of hippocampal neurons to respond normally to this hormone.

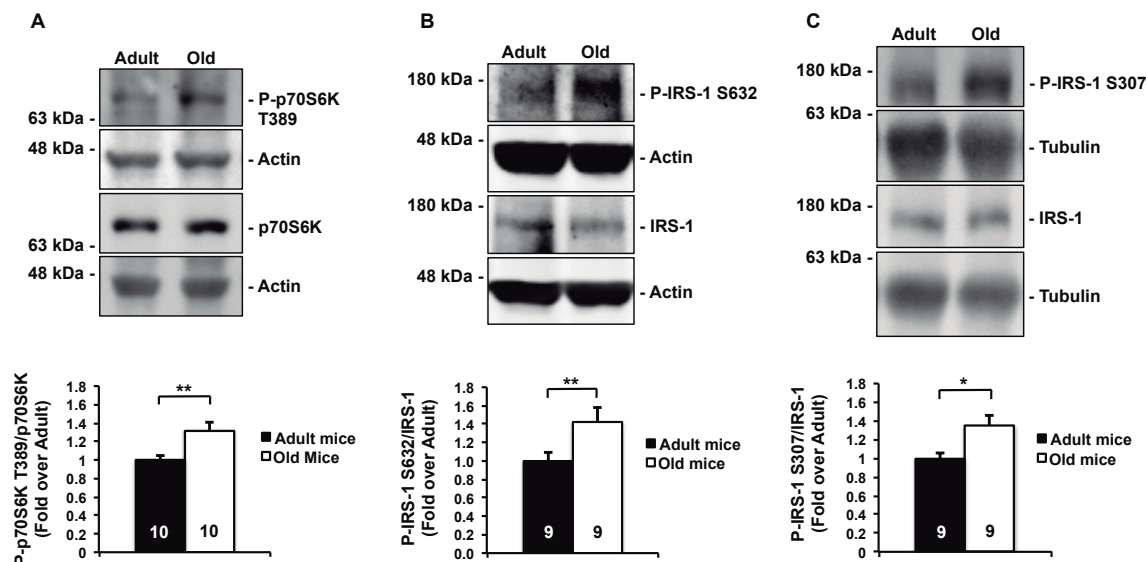


**Figure R3. PI3K/Akt signalling inhibition restores Insulin-LTD in old mice hippocampus.**

(A) Graph showing Insulin-LTD induction in hippocampal slices from old mice incubated with the PI3K inhibitor Wortmannin: untreated slices (●; n=7) and treated with Wortmannin (○ grey filled; n=7). (B) Graph representing Insulin-LTD induction in old mice hippocampal slices treated with the Akt inhibitor Quercetin: untreated (●; n=7) and treated (▲; n=8). In both cases insulin 0.5  $\mu$ M was perfused in ACSF for 30 min. Wortmannin 0.5  $\mu$ M or Quercetin 20  $\mu$ M were perfused for 60 min. starting 30 min. before insulin stimulus. The black box indicates time of insulin perfusion. The grey box indicates the time of inhibitors perfusion. The kinetic curves represent the fEPSPs as % baseline. The sample traces on the right represent the electric signal at different time points: Basal, 60 min. and 240 min. Old mice traces remain the same as reflected in Fig. R1. Data are represented as mean  $\pm$  SEM (One-way Anova; \*p<0.05).

Both in intact animals and in cultured cells, different factors (i.e. chronic stimulation by insulin, free fatty acids) can lead to insulin resistance due to desensitisation and downregulation of insulin signalling (Zick et al., 2001, Shah et al., 2004). One of the main effectors of this phenomenon is ribosomal protein S6 kinase beta-1 (S6K1), also known as p70S6 kinase (p70S6K, p70-S6K). In physiological conditions, its phosphorylation by the PI3K/Akt pathway induces the phosphorylation of the insulin receptor substrate 1 (IRS-1) at certain residues, mainly Serine 632 and Serine 307 leading to the termination of the insulin signalling (Um et al., 2004). Then, in the high PI3K/Akt background of the old hippocampus (see Fig. R2), we found reasonable to expect higher activity of p70S6K and negative phosphorylation of IRS-1. To investigate this possibility, we checked the levels of activation of p70S6K and of IRS-1 negative phosphorylation in hippocampal fractions from adult and old mice. In accordance

with post-receptor pathway desensitisation, old mice presented higher p70S6K activity and IRS-1 negative marks (Serine 632 and Serine 307 phosphorylation) compared to adult mice (Fig. R4A-C).

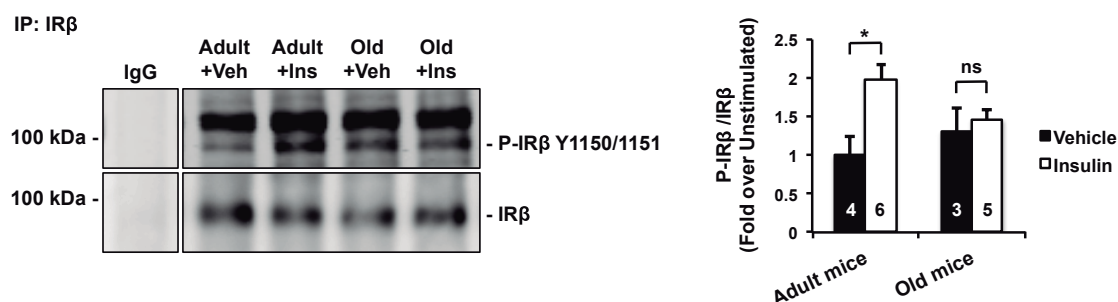


**Figure R4. Increment of insulin desensitising marks with ageing.**

(A) WB showing increased activating mark Phospho-Threonine 389 on p70S6K in hippocampal extracts from old mice. (B) and (C) Detection by WB in adult and old mice hippocampal extracts of insulin desensitising marks on the insulin receptor substrate 1 (IRS-1): Phospho-Serine 632 (centre) and Phospho-Serine 307 (right). Data are represented as mean  $\pm$  SEM (t-test for p70S6K and Ser307; Mann-Whitney U-Test for Serine 632; \* $p < 0.05$ ; \*\* $p < 0.01$ ).

### 1.3 Insulin desensitisation in old mice hippocampus is consequence of a higher basal level of receptor activity:

In the insulin desensitisation state, the serine phosphorylated IRS-1 is refractory to stimuli (Zick et al., 2001). Moreover, in this state there is also a reduction in the competence of the insulin receptor to be activated by ligand (Um et al., 2004). To further prove that the ability of the hippocampus to sense the insulin signalling is compromised with age, we injected adult and old mice intraperitoneally with 27 IU/Kg body weight of insulin. One hour after injection we extracted the hippocampus and we measured the level of receptor's activity (reflected by receptor tyrosine phosphorylation). In agreement with insulin's pathway desensitisation in the old, only in injected adult mice the exogenous insulin led to strong receptor activation, while the response in old mice was minimal (Fig. R5).



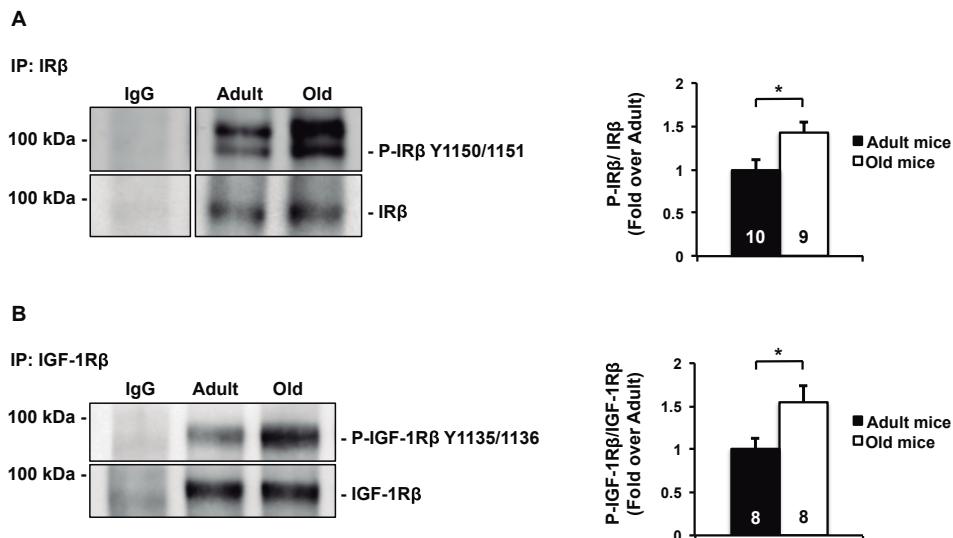
**Figure R5. Reduced insulin sensitivity in the hippocampus of old mice.**

Adult and old mice were injected with saline solution (0.9% NaCl; Veh) or insulin 27IU/kg body weight (Ins) intraperitoneally and sacrificed after 1 hour. Hippocampal IRβ receptor was immunoprecipitated followed by detection of levels of activity by western blotting using the IRβ Phospho-Tyrosine 1150/1151 antibody. Data are represented as mean ± SEM (Two-way Anova with Bonferroni's adjustment; \* $p < 0.05$ ; ns=not significant).

Previous publications from our group described that age increases the level of activity of the TrkB receptor, (Martin et al., 2008), a protein of the same receptor tyrosine kinase (RTK) family as IR and IGF-1R. The lack of receptor response to insulin stimulus in the hippocampus and the desensitisation observed in the pathway, together with these previous publications, prompted us to explore the possibility that IR basal activity would be increased in the old hippocampus. In order to make this study more comprehensive we also analysed the basal level of activity of the IGF-1 receptor, which shares downstream effectors with the IR and can also induce IRS-1 protein inhibition and therefore insulin desensitisation when persistently activated (reviewed in White, 2002; Dávila et al., 2008, Garwood et al., 2015). Consistent with the view that age affects both IR and IGF-1R-mediated signalling, we observed higher levels of active IR and IGF-1R in hippocampal membranes from old mice compared to adult mice (Fig. R6A-B), suggesting that desensitisation comes, to a certain extent, from the constitutive and high activity of the insulin and insulin-like growth factor-1 pathways.

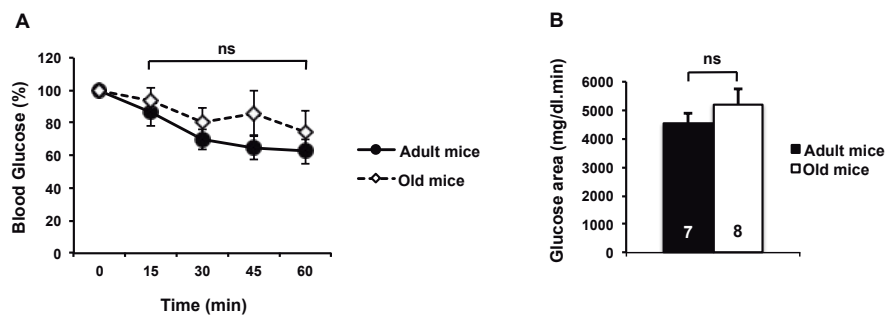
Insensitive insulin receptor and desensitised post-receptor activity are typical features of T2DM and are also observed in pre-diabetic conditions in old individuals, rising the possibility that hippocampal insulin desensitisation in the hippocampus of old mice is the brain manifestation of systemic insulin resistance. To address this possibility, we assessed whether our old mice suffered from a pre-diabetic condition or not by performing the insulin tolerance test. In this test, insulin is injected intraperitoneally (1 IU/Kg body weight) after 4 hours of fasting and then blood glucose content is measured at different time points. The result of this experiment revealed that the old mice utilised in our studies had a slight but non-signifi-

cant reduced capacity to clear glucose from the blood (Fig. R7A-B), suggesting that hippocampal insulin resistance is, to a significant extent, a brain autonomous process.



**Figure R6. High basal levels of IR $\beta$  and IGF-1R $\beta$  activity in the hippocampus of old mice.**

(A) Detection of IR $\beta$  basal activity in adult and old mice hippocampus. The activity mark Phospho-Tyrosine 1150/1151 was detected by western blotting after immunoprecipitation of total IR $\beta$  in hippocampal extracts of adult and old mice. (B) IGF-1R $\beta$  basal activity detection in adult and old mice. The activity mark Phospho-Tyrosine 1135/1136 was detected by western blotting after total receptor immunoprecipitation. Data are represented as mean  $\pm$  SEM (t-test; \* $p$ <0.05).



**Figure R7. Old mice hippocampal insulin desensitisation is independent of systemic insulin resistance.**

Insulin Tolerance Test (ITT) performed in 4 hours fasted adult and old mice. After a time zero measure of blood glucose, mice were injected with Insulin 1IU/kg body weight intraperitoneally and blood glucose was measured every 15 min. during 1 hour. Measures are represented in percentage respect to the 0 point (A) or as area under the curve (B). Data are represented as mean  $\pm$  SEM (Two-way Anova with Bonferroni's adjustment; t-test for area under the curve; ns=not significant).

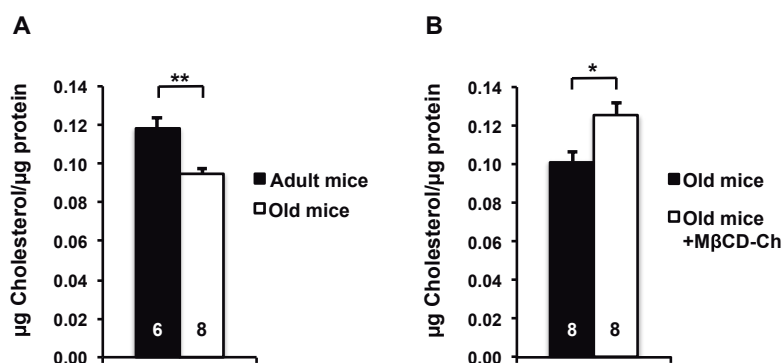
## 2. Cholesterol addition rescues insulin pathway upregulation and desensitisation

### 2.1 Age-associated hippocampal cholesterol loss plays a role in hippocampal insulin resistance:

The maintenance of the integrity and the optimal properties of the plasma membrane are an absolute prerequisite for the proper function of cells. In addition to their well known role in the maintenance of cell architecture and control of ion permeability, it is now well known their important role in the regulation of cell growth and adhesion, neurotransmission, biosynthesis of biomolecules, regulation of enzyme activity and intracellular signalling (Ginsberg et al., 1982; Goldberg et al., 1986; Krischer et al., 1993; Glynn et al., 2013; MacDonald et al., 2015). Numerous works have demonstrated that the lipid composition of neurons' plasma membrane changes with age, both in animal experimental models and in humans (reviewed in Martin et al., 2014a; Colin et al., 2016) and also in the context of insulin resistance and T2DM (Bryszewska et al., 1986; Watala et al., 1990; Bakan et al., 2006). Most significant is the reduction in hippocampal (synaptic) plasma membrane cholesterol that occurs with age (Sodero et al., 2011; reviewed in Colin et al., 2016). Hippocampal cholesterol reduction with age impairs NMDA-mediated LTD and NMDA-mediated gene expression (Martin et al., 2014b; Palomer et al., 2016a). Hence, constitutive decrease of cholesterol in the hippocampus of mice could play a role in hippocampal insulin function deficiency. In agreement with previous findings, the cholesterol levels were 20% reduced in hippocampal membrane extracts from old mice compared to adult mice (Fig. R8A).

**Figure R8. Hippocampal cholesterol levels decrease with age.**

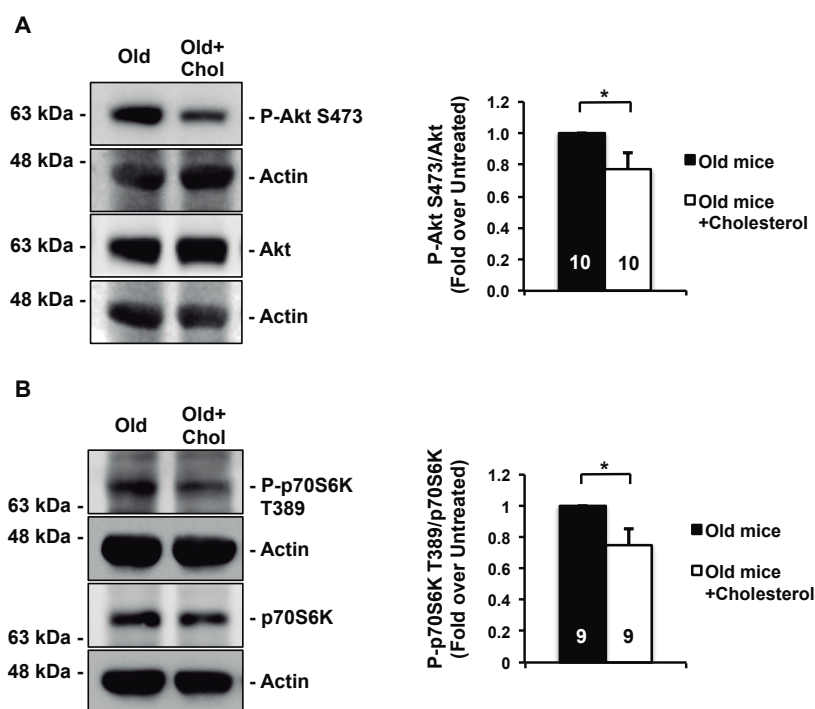
(A) Plot showing cholesterol quantification in adult and old mice hippocampal samples. (B) Quantification of cholesterol levels in old mice hippocampal slices after replenishment with a Methyl- $\beta$ -cyclodextrin-cholesterol (M $\beta$ CD-Ch) mix (30  $\mu$ M and 5  $\mu$ M, respectively). Data are represented as mean  $\pm$  SEM (t-test; Mann-Whitney U-Test for (B); \* $p$ <0.05; \*\* $p$ <0.01).



To investigate the implication of the age-associated cholesterol loss in insulin desensitisation with age, we incubated hippocampal slices from old mice with exogenous cholesterol, using Methyl- $\beta$ -cyclodextrin-cholesterol (M $\beta$ CD-Ch) at 30  $\mu$ M cholesterol for one hour (see materials and methods). This treatment restored cholesterol content back to 95%, just slightly lower than the levels of adult mice (Fig. R8B). Having established the rescue value of exogenous cholesterol, we next investigated whether cholesterol addition was sufficient for attenuating the PI3K/Akt increased signalling observed in the non-treated old (see Fig. R2). Consistent with a true functional association, cholesterol addition reduced the levels of Akt phosphorylation at Serine 473, and also the activating mark on the downstream protein in the cascade p70S6K Threonine 389 (Fig. R9A-B).

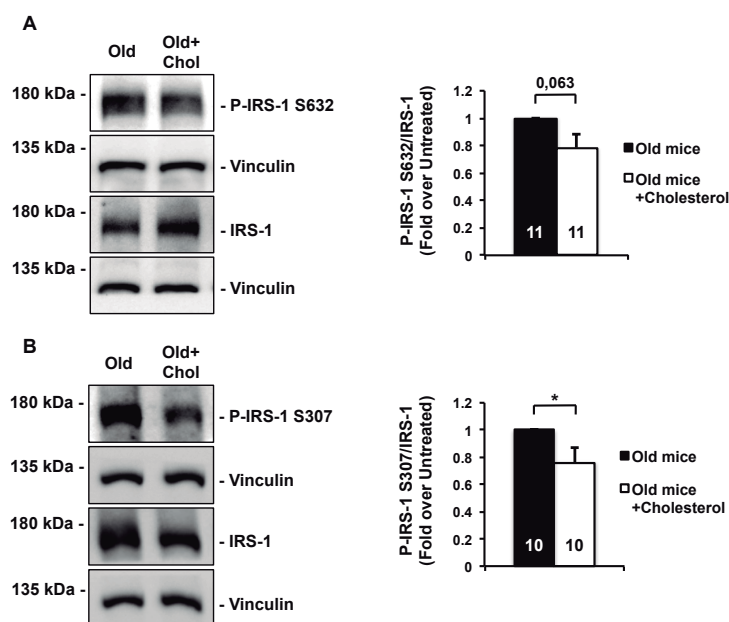
**Figure R9. Cholesterol replenishment reduces Akt signalling in old mice hippocampal slices.**

(A) Detection by WB of Akt Phospho-Serine 473 activating mark in old mice hippocampal slices replenished with cholesterol using the M $\beta$ CD-Ch mix (see Fig. R8). (B) Levels of p70S6K activity determined by Phospho-Threonine 389 activating mark in old mice hippocampal slices treated with the M $\beta$ CD-Ch mix. Data are represented as mean  $\pm$  SEM (t-test; \* $p$ <0.05).



## 2.2 Cholesterol addition rescues IRS-1 desensitisation and Insulin-LTD:

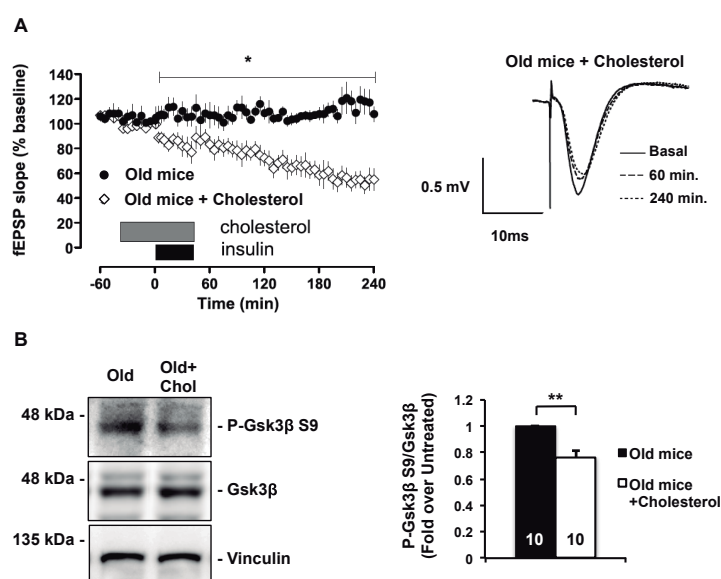
To confirm that the cholesterol-based attenuation of PI3K/Akt phosphorylation was functionally relevant (i.e. permitted insulin action), we next performed two types of experiments; biochemical and electrophysiological. Biochemically, the desensitisation marks observed in old mice were reduced upon cholesterol increase. Fig. R10A-B shows that the addition of cholesterol to old mice hippocampal slices reduced the levels of Serine 632 and Serine 307 phosphorylation on IRS-1.



**Figure R10. Insulin desensitising marks are reduced upon cholesterol addition.**

(A) WB showing reduced levels of IRS-1 Phospho-Serine 632 in old mice hippocampal slices after M $\beta$ CD-Ch treatment. (B) Levels of IRS-1 Phospho-Serine 307 desensitising mark detected by WB in old mice hippocampal slices upon cholesterol replenishment. Data are represented as mean  $\pm$  SEM (t-test; \* $p < 0.05$ ).

For the electrophysiology validation, we incubated hippocampal slices from old mice with the cholesterol-cyclodextrin mix (see above for the rescue value of this strategy) prior to the incubation with insulin and then recorded electrical activity for 4 hours as usual. Confirming that cholesterol loss plays a role in the poor insulin function in the old, the cholesterol addition allowed insulin to elicit LTD in old mice with an intensity similar to that of insulin in adult mice (Fig. R11A). Concomitantly with the rehabilitation of the synaptic plasticity signaling, the levels of the inhibitory mark Serine 9 on Gsk3 $\beta$ , protein required for the crucial LTD step of GluR2 (AMPA) internalisation, were reduced after cholesterol addition (Fig. R11B). Altogether, these last series of results indicate that one of the mechanisms by which age impairs the proper functioning of insulin in the hippocampus is through the loss of minimal but significant amounts of cholesterol.



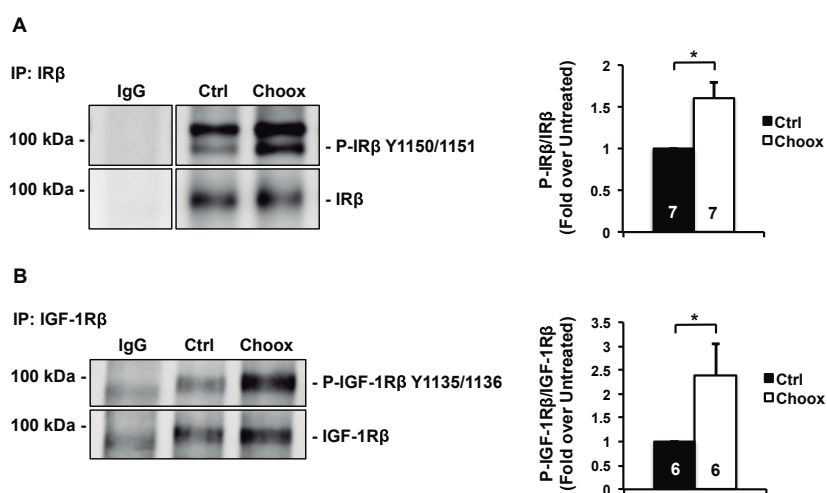
**Figure R11. Cholesterol addition rescues Insulin-LTD in old mice hippocampus.**

(A) Graph showing Insulin-LTD induction in old mice slices (●;  $n=7$ ) and old mice slices perfused with M $\beta$ CD-Ch (empty ◇;  $n=9$ ). Insulin 0.5  $\mu$ M was perfused in ACSF for 30 min. The M $\beta$ CD-Ch mix was perfused for 60 min. starting 30 min. before insulin stimulus. The black box indicates time of insulin perfusion. The grey box indicates the time of M $\beta$ CD-Ch perfusion. The kinetic curves represent the fEPSPs as % baseline. Legend continues at the bottom of next page.

### 3. Cholesterol loss is sufficient to induce insulin pathway desensitisation

#### 3.1 Cholesterol loss causes insulin pathway upregulation:

To further assess the role of age-associated cholesterol loss in brain insulin resistance with age we performed a series of sufficiency experiments: i.e. removed a small amount of cholesterol to young neurons followed by the analysis of insulin signalling. The prediction was that if the levels of this sterol were involved, a reduction in cholesterol content would lead to the over-stimulation of the insulin and IGF-1 pathways.



**Figure R12. Cholesterol reduction increases IRβ and IGF-1Rβ basal activity in hippocampal neurons in culture.**

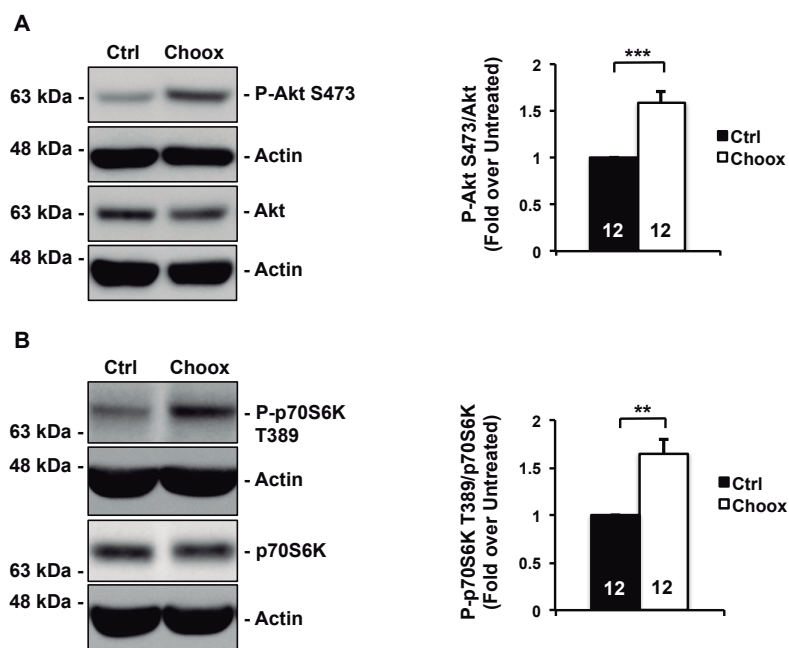
(A) Detection of active IRβ in cultured neurons before and after cholesterol oxidase (Choox) treatment. WB of activity levels (determined by Phospho-Tyrosine 1150/1151 residues) in IRβ after immunoprecipitation with total IRβ antibody. (B) Levels of activity residues in IGF-1Rβ (Phospho-Tyrosine 1135/1136) after immunoprecipitation with total IGF-1Rβ antibody. Choox treatment was 10 IU/ml for 30 min. Data are represented as mean ± SEM (t-test; \*p<0.05).

We first analysed the levels of activity of the IR/IGF-1R under reduced cholesterol conditions in cultured hippocampal neurons. Endogenous cholesterol was reduced by the addition of 10 IU/ml *Cholesterol oxidase* (Choox) for 30 min. This protocol has been previously used allowing us to achieve a significant reduction of plasma membrane cholesterol without affecting cell viability (Brachet et al., 2015; Palomer et al., 2016b). Consistent with a role in insulin resistance, the reduction of cholesterol led to an increase in IR/IGF-1R activity (Fig. R12A-B). Next, we checked the effect of cholesterol removal on other members of the signalling cascade. As shown in Fig. R13A-B, cholesterol removal effectively induced the activation of the downstream IR/IGF-1R effectors Akt and p70S6K.

**Figure R11.** The sample traces on the right represent the electric signal at different time points: Basal, 60 min. and 240 min. Old mice traces remain the same as reflected in Fig. R1. (B) Detection by WB of Gsk3β Phospho-Serine 9 inhibiting mark in old mice hippocampal slices replenished with cholesterol using MβCD-Ch. Data are represented as mean ± SEM (One-way Anova for (A); t-test for (B); \*p<0.05; \*\*p<0.01).

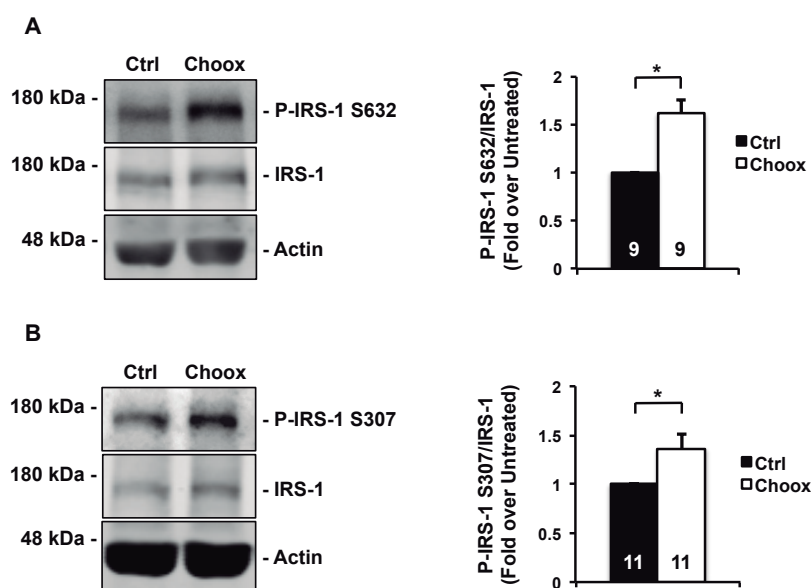
**Figure R13. Cholesterol reduction increases Akt activity.**

(A) WB reveals higher levels of Akt activity (Phospho-Serine 473), after cholesterol depletion using Choox. (B) WB reveals high levels of active p70S6K (Phospho-Threonine 389) in cultured hippocampal neurons with reduced cholesterol (Choox-treated) levels. Data are represented as mean  $\pm$  SEM (t-test; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



### 3.2 Cholesterol loss induces insulin desensitising marks:

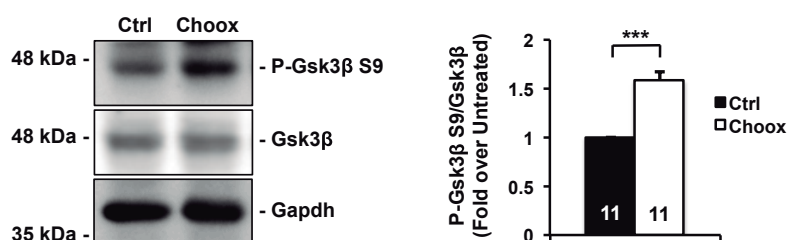
Considering that both receptors, insulin and IGF-1, share post-receptor effectors, the increased activity of IR and IGF-1R in the low cholesterol/ageing condition suggests that this could be the mechanism underlying age-associated insulin desensitisation. In order to validate this assumption, we checked the levels of insulin desensitising marks in neurons with reduced cholesterol.



**Figure R14. Cholesterol reduction increases the levels of insulin desensitising marks.**

(A) WB of IRS-1 Phospho-Serine 632 desensitising mark levels in cultured neurons after Choox treatment. (B) WB of IRS-1 Phospho-Serine 307 desensitising mark in hippocampal neurons in culture after cholesterol reduction. Data are represented as mean  $\pm$  SEM (t-test; \* $p < 0.05$ ).

Consistent with the prediction, cholesterol reduction led to higher levels of IRS-1 Serine 632 and Serine 307 phosphorylation (Fig. R14A-B). To determine if the cholesterol removal affects the capability of young neurons to respond with LTD to insulin application, we analysed the activity of Gsk3 $\beta$ , a key determinant of the GluR2 internalisation needed for LTD. In agreement with this possibility, the reduction of cholesterol led to higher levels of inhibited Gsk3 $\beta$  (Fig. R15).

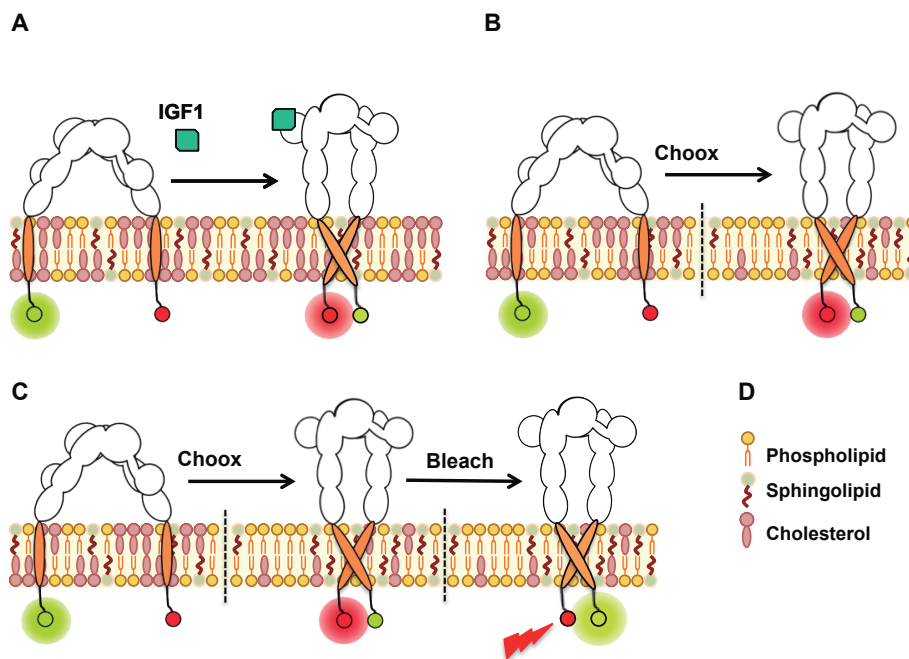


**Figure R15. Increased levels of Serine 9 Gsk3 $\beta$  inhibiting mark after cholesterol reduction.**

WB of Gsk3 $\beta$  Phospho-Serine 9 levels in cultured hippocampal neurons after Chox treatment. Data are represented as mean  $\pm$  SEM (t-test; \*\*\*p < 0.001).

### 3.3 Cholesterol loss allows spontaneous activation of insulin/IGF-1 receptors:

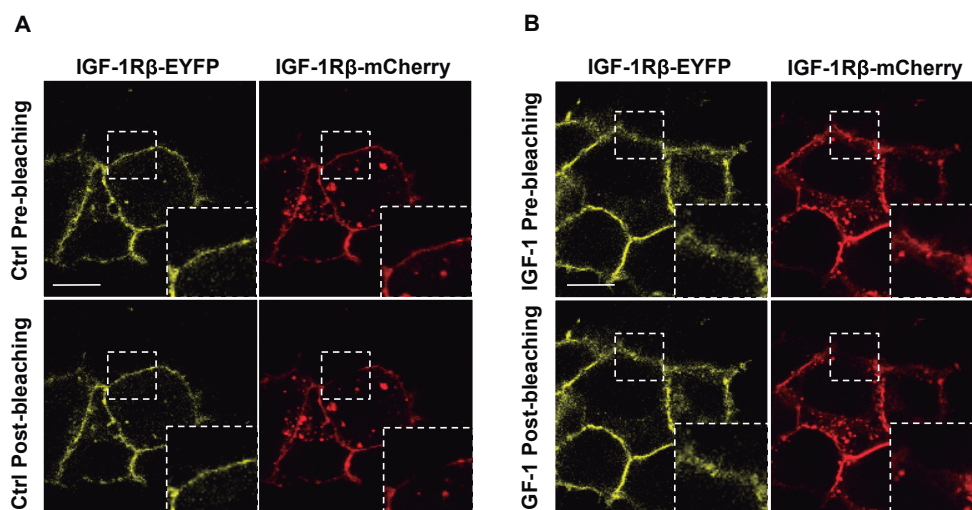
Next, we investigated the mechanism by which the loss of cholesterol leads to the activation of the insulin and IGF-1 pathways. As mentioned in the Introduction both insulin and IGF-1 receptors are very similar in structure and activate the same downstream signalling pathway. Moreover, in order to become active both receptors require insulin or IGF-1 binding to their extracellular domain, inducing a conformational change in their transmembrane domain which leads to the physical approaching and autophosphorylation of their cytoplasmic tails (Hubbard et al., 1994 and 1997; Favelyukis et al., 2001; Lemmon et al., 2010; Kavran et al., 2014). Therefore, we speculated that the cholesterol loss observed in old mice could induce insulin/IGF-1 receptor subunits approximation and activation, in a manner similar to that induced by ligands. In order to test this possibility, we used full-length IGF-1R constructions bearing fluorescent donor or acceptor tags in the intracellular domain (as in Kavran et al., 2014). We performed FRET efficiency analysis of these constructs co-transfected in Hek 293T cells, following a previously established protocol (see materials and methods and Kavran et al., 2014). The experimental design is shown in Fig. R16A-D.



**Figure R16. Simplified scheme of acceptor-photobleaching experiments.**

FRET experiments are based on the capacity of one of the fluorophores of the pair to transfer energy to the other, also call FRET efficiency. In our experiments we transfected plasmids coding for IGF-1R $\beta$  sequence modified through the addition at the cytosolic/kinase domains of a yellow (EYFP) or a red (mCherry) fluorophore-coding sequence. (A) Conventional FRET response in the presence of ligand: induction of cytosolic domain approximation leading to increased FRET efficiency (transmembrane approximation leads to cytoplasmic approximation and autophosphorylation). (B) Possible mechanism of cholesterol loss: cholesterol level reduction at the plasma membrane (right side of the dotted line) induces a conformational change in the IGF-1R $\beta$  allowing the approximation of the kinase domains and thus increasing FRET efficiency. (C) The figure represents the FRET technique used: acceptor-photobleaching FRET. In this kind of FRET the intensity of donor fluorophore is measured after bleaching the acceptor. If there was FRET and energy was being transferred, the bleaching of the acceptor signal will increase the intensity of the donor signal and FRET efficiency will be higher. The diagrams are adapted from Kavran et al. 2014. (D) Lipids legend.

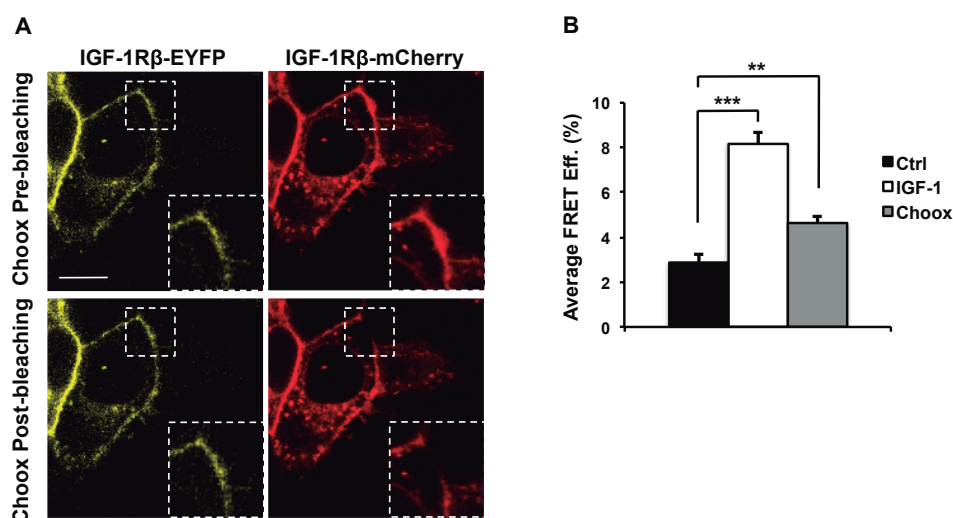
We first determined the basal levels of FRET efficiency in transfected Hek 293T cells. We used the acceptor-photobleaching FRET where the approximation of the fluorescent molecules and transference of energy between them is measured by the increment of donor intensity upon acceptor photobleaching (see materials and methods). Fig. R17A shows the basal FRET efficiency (quantified in Fig. R18B). The addition of IGF-1 to transfected Hek 293T cells caused an increase in the FRET efficiency, as reflected by the increment in the donor signal after acceptor photobleaching (Fig. R17B). It is important to note that the increase in FRET indicates the receptor's cytosolic domain approximation and that this is a *sine qua non* condition for autophosphorylation (Murakami et al., 1991; Hubbard et al., 1994). Hence, FRET increases indicate receptor activation.



**Figure R17. IGF-1 addition increases FRET efficiency of donor-acceptor fluorophore pair coupled to IGF-R $\beta$ .**

(A) and (B) Laser microscopy images of Hek 293T cells co-transfected with IGF-1R $\beta$ -EYFP (donor fluorophore) and IGF-1R $\beta$ -mCherry (acceptor fluorophore), with the fluorophores replacing the cytosolic tails of the entire receptor sequence. Cells were starved for 5 hours and remained unstimulated (A) or were stimulated with 4  $\mu$ M IGF-1 for 25 min. (B) before fixation. Acceptor signal was bleached, intensity of donor signal was measured and FRET efficiencies were calculated. Plots of FRET efficiencies are specified in Fig. R18. Scale bars represent 10  $\mu$ m.

In this regard, removal of cholesterol in the absence of ligand led to an increase in FRET efficiency in the transfected Hek cells (Fig. R18A). The energy transfer obtained in the reduced cholesterol background was significant yet smaller than the increase achieved by the natural ligand (Fig. R18B). This is an expected outcome, consistent with insulin resistance developing slowly, in parallel with the slow progression of brain cholesterol changes.

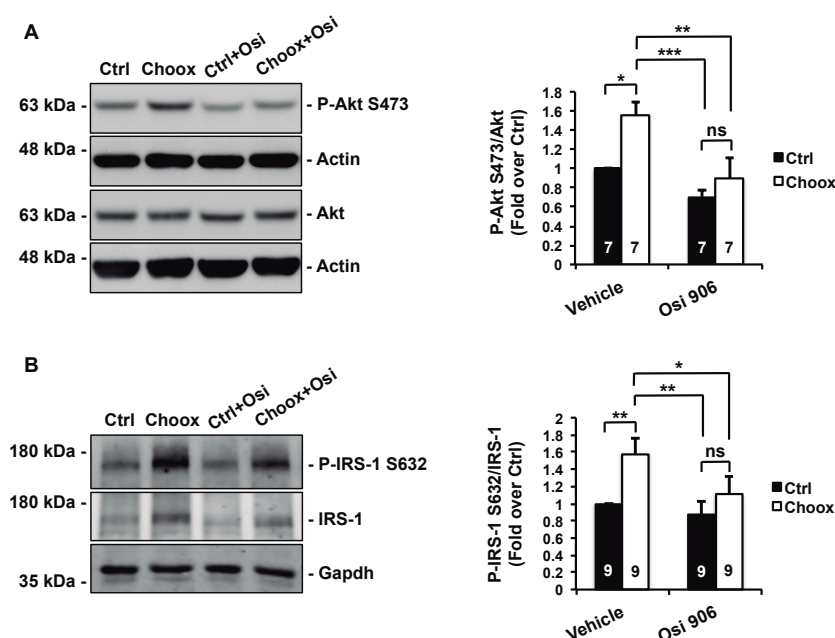


**Figure R18. Cholesterol levels reduction increases FRET efficiency of donor-acceptor fluorophore pair coupled to IGF-1R $\beta$ .**

*Legend continues at the bottom of next page.*

### 3.4 Cholesterol loss-mediated receptor activation determines pathway desensitisation in hippocampal neurons:

In order to give neuronal “weight” to the Hek 293T cells’ results, we investigated the contribution of cholesterol-induced receptor activation in cultured hippocampal neurons. To address this possibility, low cholesterol-induced IR and IGF-1R activation (see Fig. R12) were incubated in the presence of the OSI-906 inhibitor, an ATP competitive inhibitor that avoids autophosphorylation of the IR and IGF-1R (Mulvihill et al., 2009). Inhibition of both receptors caused a significant, but not complete, reduction in the activation of Akt (Fig. R19A). Moreover, OSI-906 induced a significant reduction on Serine 632 phosphorylation of the IRS-1 (Fig. R19B). Altogether these results reinforce the view that age-associated cholesterol loss participates in insulin desensitisation by, to a large extent, increased IR/IGF-1R activation.



**Figure R19. IR $\beta$  and IGF-1R $\beta$  activities are required for cholesterol removal-mediated high Akt activity and insulin desensitisation.**

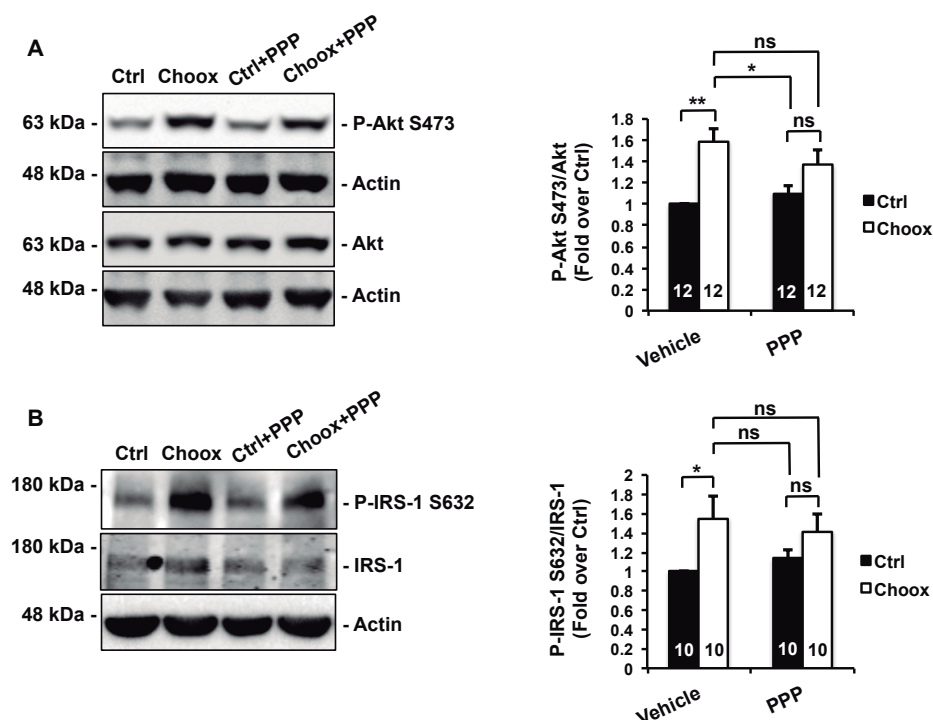
(A) Cultured hippocampal neurons were pre-treated for one hour, previous to Choox treatment, with IR $\beta$  and IGF-1R $\beta$  inhibitor OSI-906 (named Osi; 1  $\mu$ M). Image shows detection by WB signal of Akt Phospho-Serine 473 activating mark. (B) WB of IRS-1 Phospho-Serine 632 desensitising mark in cultured neurons, treated or not with OSI-906 previous to cholesterol removal with Choox. Data are represented as mean  $\pm$  SEM (One-way Anova with Bonferroni's adjustment; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; ns=not significant).

**Figure R18.** (A) Laser microscopy images of Hek 293T cells co-transfected with IGF-1R $\beta$ -EYFP (donor fluorophore) and IGF-1R $\beta$ -mCherry (acceptor fluorophore), with the fluorophores replacing the cytosolic tails of the receptor. Cells were starved for 5 hours and then treated with 40 IU/ml Choox for one hour before fixation. Acceptor-photobleaching technique was performed, measuring the donor intensity increment that would reflect a higher FRET efficiency. Scale bar represents 10  $\mu$ m. (B) Fig. R17 and Fig. R18 images' quantification. Average FRET efficiency values for acceptor-photobleaching are plotted for each of the conditions: Control or unstimulated, treated with IGF-1 ligand or with Choox. The graphic represents data obtained from 120 images quantified of three independent experiments for each condition. FRET efficiency was calculated as  $FRET\ Efficiency\ (\%) = [(Donor_{post} - Donor_{pre}) / Donor_{post}] \times 100$ . Data are represented as mean  $\pm$  SEM (Kruskal-Wallis test with Dunn's adjustment; \*\* $p$ <0.01; \*\*\* $p$ <0.001).

#### 4. Additional mechanistic insights

##### 4.1 IGF-1R inhibition alone is not enough for reducing insulin desensitisation:

Regarding the demonstration that insulin desensitisation with age implies IR/IGF-1R activation by a cholesterol loss-mediated mechanism, we next checked the individual contribution of IR and IGF-1R. First, we checked the effect of Picropodophyllin (PPP), the first specific IGF-1R inhibitor described not to interfere with IR (Girnita et al., 2004), in the Choox treated neurons. This inhibitor avoids substrate phosphorylation and autophosphorylation of the receptor. As shown in Fig. R20A exclusive inhibition of IGF-1R only moderately reduced the activation of Akt induced by cholesterol reduction. Consequently with the partial reduction of Akt signalling, the levels of the desensitising mark Serine 632 on IRS-1 were moderately reduced after cholesterol loss in the PPP treated cells (Fig. R20B). These results suggest the requirement of both receptors signalling, IR and IGF-1R, in the upregulation of the downstream pathway and posterior desensitisation through specific marks.

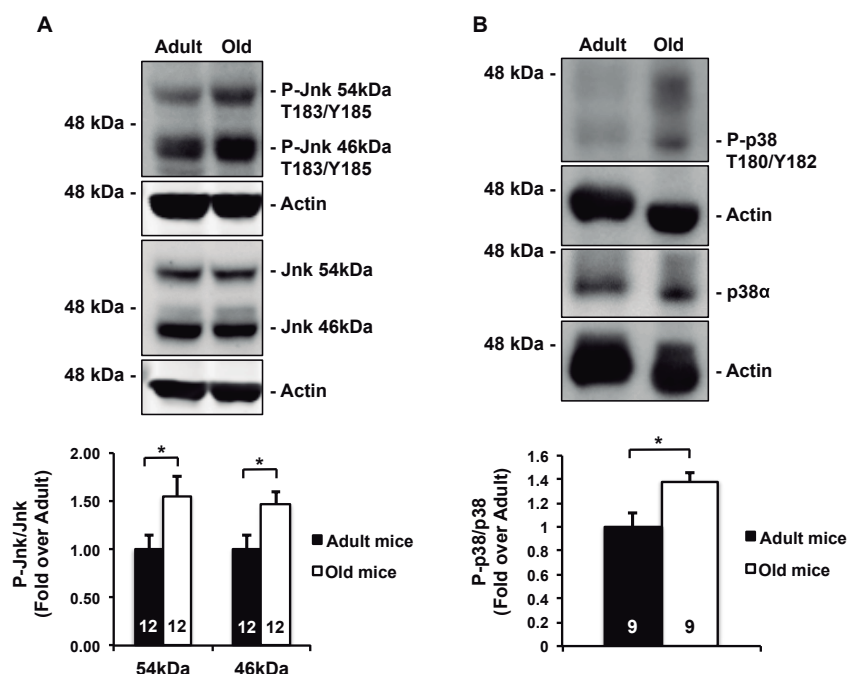


**Figure R20. The inhibition of IGF-1R $\beta$  alone is not sufficient to avoid cholesterol loss-mediated increase in Akt activity and insulin desensitisation.**

(A) WB of Phospho-Serine 473 Akt activating mark after IGF-1R $\beta$  inhibition with PPP (1  $\mu$ M, 1 hour treatment) in reduced cholesterol neurons. (B) WB of PPP inhibitory effect on insulin desensitising mark IRS-1 Phospho-Serine 632 after cholesterol removal with Choox. Data are represented as mean  $\pm$  SEM (Friedman test with Dunn's adjustment; \* $p$ <0.05; \*\* $p$ <0.01; ns=not significant).

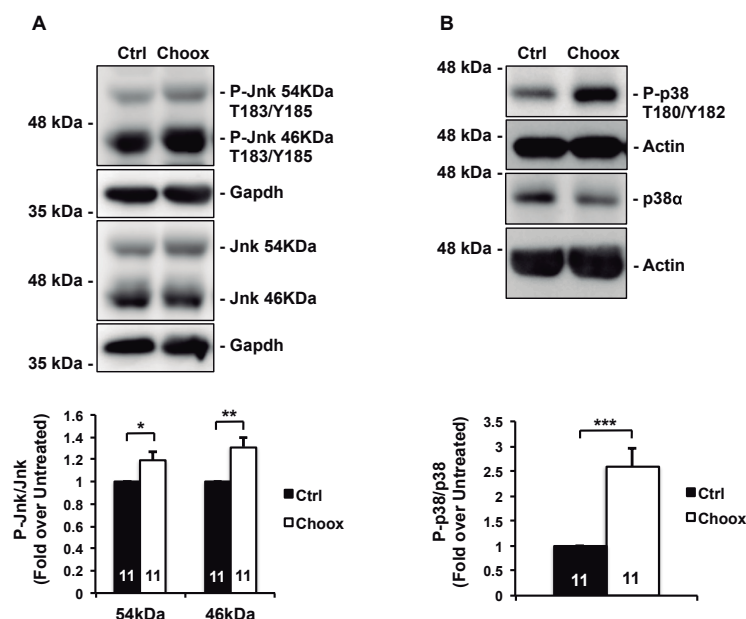
#### 4.2 Increased inflammation-associated signalling does not play a (major) role in reduced Insulin-LTD of the old:

Although we present a series of evidences consistent with the view that much of the insulin pathway desensitisation comes from receptor chronic activation of the PI3K/Akt pathway, and by this mean the activation of the negative feedback loop leading to IRS-1 Serine 307 and Serine 632 phosphorylation, insulin pathway desensitisation in the old hippocampus might have other origins. In this sense, and most remarkable in the ageing context, are the several publications describing the occurrence of desensitising marks on the IRS-1 associated with inflammatory activity. A main target is however Serine 307 phosphorylation on IRS-1, which is performed mainly by Jnk and also potentiated by others pro-inflammatory proteins like IKK $\beta$  (Aguirre et al., 2000; Yuan et al., 2001; Hirosumi et al., 2002; Tian et al., 2014). Also, inflammation-related increase in p38 activity has been related with insulin desensitisation (Jiang et al., 2004; Pereira et al., 2016; Nandipati et al., 2017). In view that both Jnk and p38 activities increase in the brain with age (Hu et al., 2006; Zhou et al., 2009; Li et al., 2011; Orejana et al., 2013), we explored the possibility that the activation in aged mice of these pathways could have also contributed to the insulin desensitising signals observed in old mice. In support of this possibility the activity of Jnk and p38 were significantly increased in old mice hippocampus *in vivo* (Fig. R21A-B).



**Figure R21. High activity of the MAPKs Jnk and p38 in ageing mice hippocampus.**

(A) WB of Jnk activity levels (Phospho-Threonine 183 and Tyrosine 185) in hippocampal extracts from adult and old mice. (B) WB of p38 activating marks Phospho-Threonine 180 and Tyrosine 182 detected in hippocampal extracts from adult and old mice. Data are represented as mean  $\pm$  SEM (t-test for 54kDa Jnk and p38; Mann-Whitney U-Test for 46kDa; \* $p < 0.05$ ).



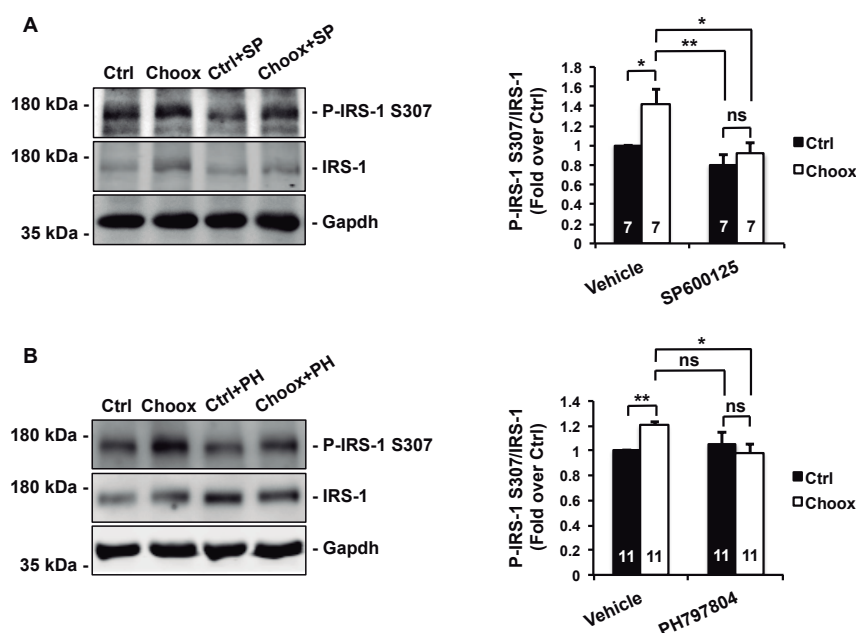
**Figure R22. Jnk and p38 activation is increased in cultured hippo-campal neurons after cholesterol removal with Choox.**

(A) WB of Jnk activating marks Phospho-Threonine 183 and Tyrosine 185 in cultured hippocampal neurons after cholesterol removal with Choox. (B) WB of p38 activating marks Phospho-Threonine 180 and Tyrosine 182 detected in cultured hippocampal neurons after Choox treatment. Data are represented as mean  $\pm$  SEM (t-test; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001).

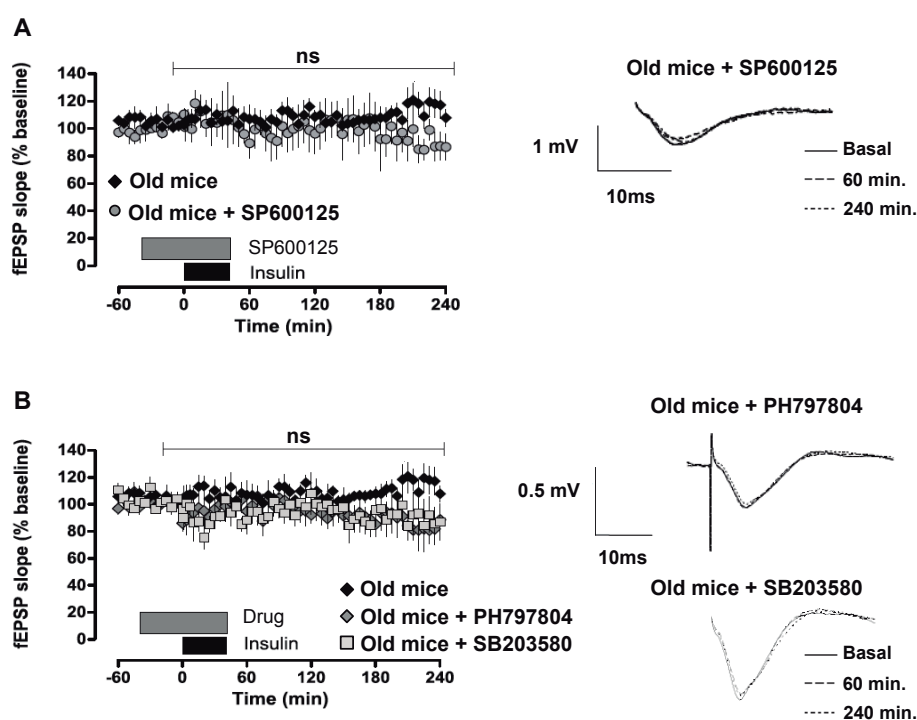
To test if these enzymes' increased activity with age was related to the cholesterol loss that occurs during ageing, cultured hippocampal neurons were subjected to Choox treatment followed by SDS PAGE and western blotting. Consistent with a cause-effect relationship, the cholesterol reduction treatment revealed a significant increase in Jnk and p38 activities *in vitro* (Fig. R22A-B). Therefore, we next checked if the inhibition of p38 and Jnk activity played a role in the increase in Serine 307 phosphorylation that occurs in response to the cholesterol reduction treatment.

**Figure R23. Inhibition of Jnk and p38 prevents the insulin desensitising mark on IRS-1.**

(A) WB of insulin desensitising mark IRS-1 Phospho-Serine 307 in cultured hippocampal neurons. Neurons were treated with the Jnk inhibitor SP600125 (named SP; 10  $\mu$ M, 1-hour treatment) before cholesterol reduction through Choox. (B) Levels of IRS-1 Phospho-Serine 307 in neurons treated with the p38 inhibitor PH797804 (named PH; 2  $\mu$ M, 1 hour treatment) before cholesterol levels reduction through Choox. Data are represented as mean  $\pm$  SEM (One-way Anova with Bonferroni's adjustment; \* $p$ <0.05; \*\* $p$ <0.01; ns=not significant).



Consistent with this possibility, the inhibition of Jnk and p38 caused a reduction in Serine 307 phosphorylation levels in cultured hippocampal neurons (Fig. R23A-B). In order to determine the contribution of high Jnk and p38 activities in the poor insulin signalling of the old, we tested by electrophysiological means the effect of inhibiting these kinases' activities. As shown in Fig. R24A-B, the inhibition of either Jnk or p38 did not result in the restoration of Insulin-LTD in hippocampal slices from old mice. These results suggest that the activation of inflammatory signalling, despite is contributing to insulin desensitisation, is not critically involved in the poor electrophysiological response to insulin in the old hippocampus. Considering the Insulin-LTD recovery using Wortmannin and Quercetin (Fig. R3A-B) these data reinforce the PI3K/Akt hyper-activation as the main event underlying poor Insulin-LTD in old mice.

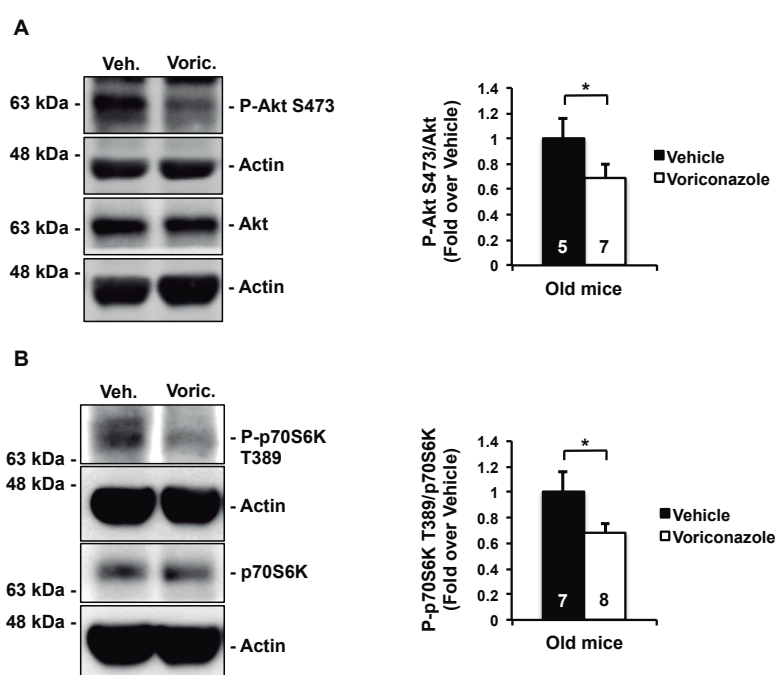


**Figure R24. Inhibition of MAPKs Jnk or p38 is not sufficient to rescue Insulin-LTD in old mice hippocampus.**

(A) Graph showing Insulin-LTD induction in old mice slices (◆; n=7) and old mice slices perfused with Jnk inhibitor SP600125 (○ grey filled; n=6). (B) Graph representing Insulin-LTD induction in old mice (◆; n=7), old mice + p38 inhibitor PH797804 (◆ grey filled; n=4) and old mice + p38 inhibitor SB203580 (□ grey filled; n=6). In both figures insulin 0.5  $\mu$ M was perfused in ACSF for 30 min. SP600125 1  $\mu$ M, PH797804 1  $\mu$ M or SB203580 0.5  $\mu$ M were perfused for 60 min. starting 30 min. before insulin stimulus. The black box indicates time of insulin perfusion. The grey box indicates the time of inhibitors' perfusion. The kinetic curves represent the fEPSPs as % baseline. The sample traces on the right represent the electric signal at different time points: Basal, 60 min. and 240 min. Old mice traces remain the same as reflected in Fig. R1. Data are represented as mean  $\pm$  SEM (One-way Anova; ns=not significant).

### 5. The CYP46A1 inhibitor Voriconazole rescues insulin desensitisation in old mice

Although not investigated in depth, age-associated cholesterol loss in the hippocampus is thought to be the consequence of a number of events, namely, i) reduced synthesis by neurons (Fünfschilling et al., 2007; Saito et al., 2009), ii) reduced synthesis in astrocytes, the main neuron-cholesterol delivering cells in the adult brain (Quan et al., 2003; Thelen et al., 2006; Nieweg et al., 2009) and, perhaps the most relevant iii) increased expression levels and activity of the cholesterol catabolic enzyme cholesterol 24-hydroxylase, or CYP46A1 (Lütjohann et al., 2000; Sodero et al., 2011). Consistent with this last possibility, the expression in the brain of this enzyme increases with age, both in rodents and in humans (reviewed in Martin et al., 2014a). The cause of the age-associated increase is not known, though the early demonstration that this enzyme is regulated at the transcriptional level by oxidative stress metabolites (Ohyama et al., 2006) would suggest that its activation in the old might obey to the same factors, which abundantly accumulate in ageing tissues. Irrespective of the underlying mechanism of this enzyme's activation, we tested its involvement in the age-associated insulin insensitivity. For this purpose, we inhibited the activity of this enzyme through the oral administration to old mice of the compound Voriconazole.



**Figure R25. Voriconazole treatment decreases Akt downstream signalling up-regulation in ageing mice hippocampus.**

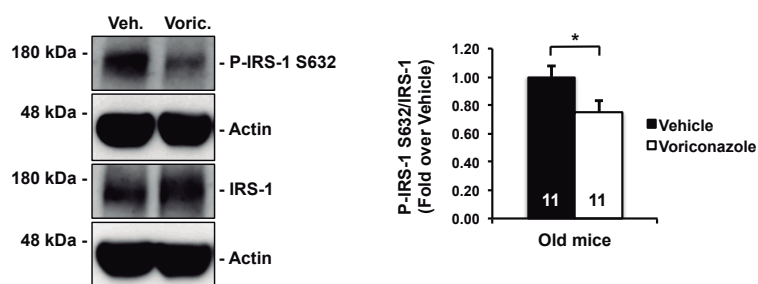
(A) WB of Akt Phospho-Serine 473 activating mark in hippocampal extracts from old mice treated during 45 days with vehicle (Veh.) or the CYP46A1 inhibitor Voriconazole (Voric.). (B) Levels of p70S6K Phospho-Threonine 389 activating mark in the same hippocampal extracts. Data are represented as mean ± SEM (t-test; \*p<0.05).

Voriconazole is a triazole antifungal drug that presents high specificity for CYP46A1, both *in vitro* and *in vivo* (Shafaati et al., 2010). Importantly, the *in vivo* effects in the brain were observed after systemic administration, implying that this compound efficiently crosses the blood-brain barrier (Shafaati et al., 2010). Moreover, and consistent with this enzyme's

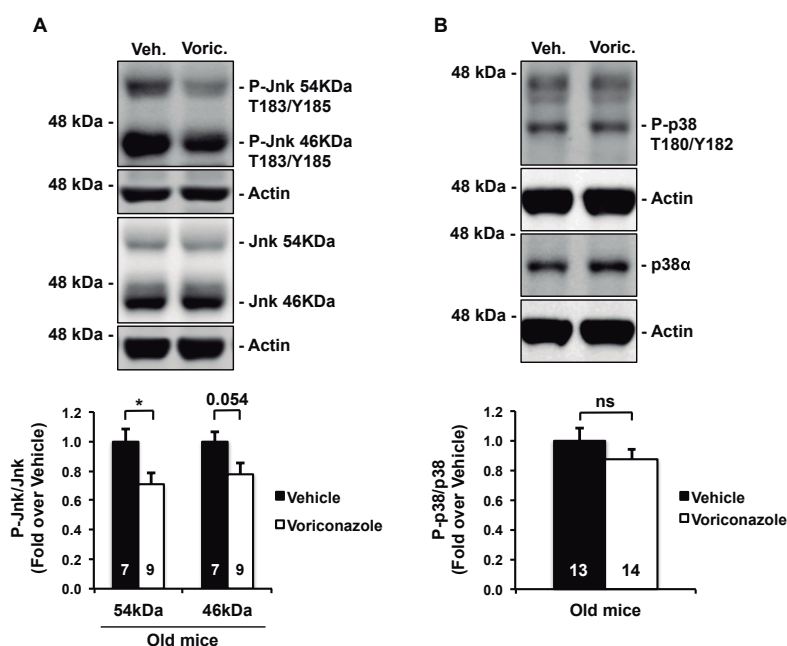
activity playing a role in the cognitive deficits of the old, a recent publication from our group revealed that the oral administration of Voriconazole to old mice prevents both, the loss of cholesterol that normally accompanies ageing and (some of) the cognitive deficits typical of this stage of life (Palomer et al., 2016b). Hence, we treated 20 month-old mice orally with a Voriconazole solution (60 mg/kg body weight/day) during 45 days and at the end of the treatment studied the effect on insulin signalling.

**Figure R26. Voriconazole treatment reduces IRS-1 Phospho-Ser632 in the hippocampus of old mice.**

WB of IRS-1 Phospho-Serine 632 desensitising mark in hippocampal extracts of vehicle (Veh.) or Voriconazole (Voric.) treated old mice. Data are represented as mean  $\pm$  SEM (t-test; \* $p < 0.05$ ).



This experimental approach results in the significant reduction of the cholesterol loss accompanying ageing (Palomer et al., 2016b). In agreement with this result and with the previous rescue effects of exogenous cholesterol, (see Figs. R9-R11) this treatment efficiently reduced Akt and p70S6K activity in the hippocampus of old mice (Fig. R25A-B) and also the Serine 632 inhibitory mark on IRS-1 (Fig. R26). In addition, we observed that Voriconazole treatment also rescued the increment in old mice of some of the inflammatory proteins' activity described to be upregulated upon cholesterol loss, like Jnk (fig. R27A).

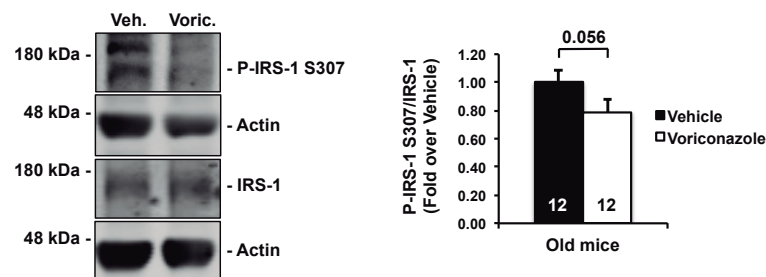


**Figure R27. Voriconazole partially attenuates Jnk and p38 MAPKs' activation in hippocampal extracts from old mice.**

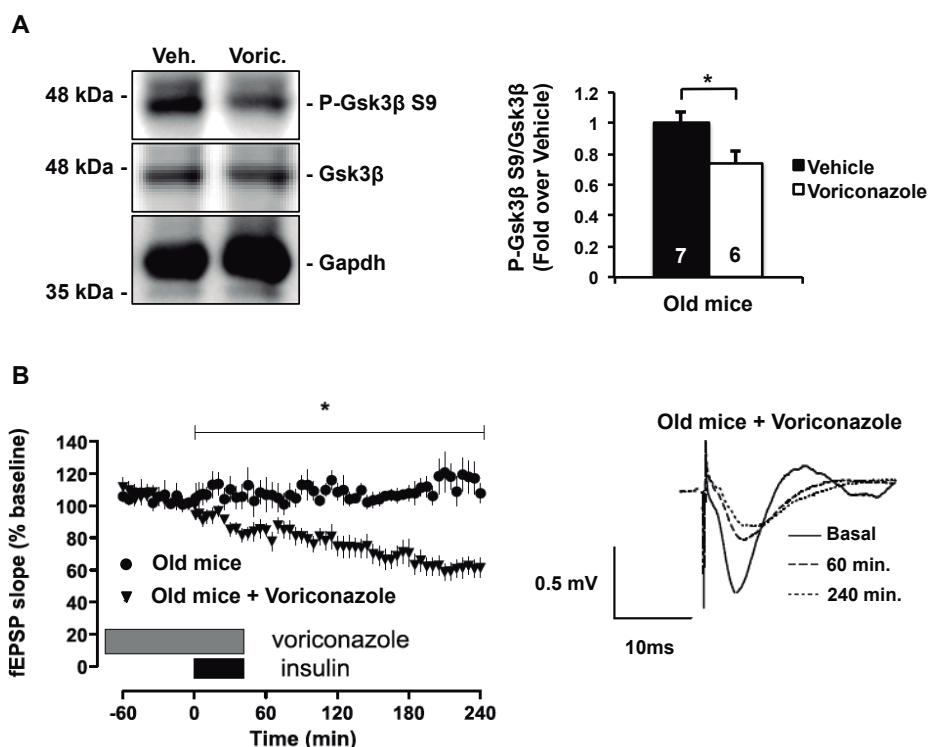
(A) WB of Jnk activating marks, Phospho-Threonine 183 and Tyrosine 185, in hippocampal extracts of old mice treated with Vehicle (Veh.) or Voriconazole (Voric.) for 45 days. (B) Levels of p38 Phospho-Threonine 180 and Tyrosine 182 activating marks, detected by WB, in the same hippocampal extracts. Data are represented as mean  $\pm$  SEM (t-test; \* $p < 0.05$ ; ns=not significant).

**Figure R28. Voriconazole treatment reduces IRS-1 Phospho-Ser307 in the hippocampus of old mice.**

Detection by WB of levels of insulin desensitising mark IRS-1 Phospho-Serine 307 in hippocampal extracts of vehicle (Veh.) or Voriconazole (Voric.) treated old mice. Data are represented as mean  $\pm$  SEM (t-test; \* $p < 0.05$ ).



However, the treatment was not able to completely revert the inflammatory effect associated to old state, as p38 activity was not significantly reduced (fig. 27B). Nevertheless, and consistent to the higher influence described of Jnk over Serine 307 resistance mark performance (Aguirre et al., 2000; Shoelson et al., 2006), the treatment with Voriconazole reduced Serine 307 desensitising mark on IRS-1 protein (Fig. R28).



**Figure R29. Voriconazole treatment rescues Insulin-LTD in hippocampal slices from old mice.**

(A) WB of Gsk3 $\beta$  Phospho-Serine 9 inhibiting mark in hippocampal extracts of old mice. Old mice were treated with Vehicle (Veh.) or Voriconazole (Voric.) (B) Graph showing Insulin-LTD induction in old mice slices (●; n=7) and old mice slices perfused with Voriconazole (▼; n=8). Insulin 0.5  $\mu$ M was perfused in ACSF for 30 min. Voriconazole 10 nM was perfused for 60 min. starting 30 min. before insulin stimulus. The black box indicates time of insulin perfusion. The grey box indicates the time of Voriconazole perfusion. The kinetic curves represent the fEPSPs as % baseline. The sample traces on the right represent the electric signal at different time points: Basal, 60 min. and 240 min. Old mice traces remain the same as reflected in Fig. R1. Data are represented as mean  $\pm$  SEM (t-test for (A); One-way Anova for (B); \* $p < 0.05$ ).

To determine to which extent the rescue effects of the Voriconazole treatment on the insulin desensitisation marks were paralleled by a reduction in the marks that could be involved in the reduced Insulin-LTD of the old, we measured the levels of phosphorylation of Serine 9 at Gsk3 $\beta$ . As mentioned, Gsk3 $\beta$  is one of the proteins implicated in the GluR2 internalisation required for the establishment of Insulin-LTD. In accordance with a close link between the reduction in the desensitisation marks and desensitisation mediators, Voriconazole treatment resulted in lower inhibitory phosphorylation of Gsk3 $\beta$  at Serine 9 (Fig. R29A). Finally, we tested if Voriconazole treatment was able to rescue the poor hippocampal Insulin-LTD of old mice. Pre-incubation of old hippocampal slices with Voriconazole was sufficient to restore the ability of insulin to elicit LTD (Fig. R29B), all in all confirming the relevance of the CYP46A1 mediated pathway in the age-associated and cholesterol loss-dependent impairment in brain insulin signalling.

# *DISCUSSION*

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### ***1. Reduced insulin signalling in the old brain: weaker plasticity in order to improve survival?***

The results in this thesis provide insights into the phenomenon of insulin and insulin-like growth factor-1 (IGF-1) signalling in the ageing brain. Due to the different and important processes these factors regulate, they have become especially relevant in ageing progression. The loss of insulin signalling, and to a great extent also IGF-1, is a known and well-described event in old organisms. In fact, reduced insulin signalling is considered a hallmark of ageing with a most important physiological role: to improve survival (Fontana et al., 2010; Laplante et al., 2012). Works in different species suggest that this ligand-dependent activity reduction allows cells to minimise the anabolic processes triggered by these pathways and thus minimise the associated detrimental effects (mainly oxidative stress). In addition, insulin and IGF-1 signalling reduction favours the activation of several supports in the survival struggle, e.g. different transcription factors expression associated to stress response like FOXO1 (Van der Horst et al., 2007; Cohen et al., 2009). Besides, it leads to autophagy activation, a process implicated in the degradation of old and damaged organelles thus improving cell performance (Bjedov et al., 2010; López-Otín et al., 2013; Li et al., 2016).

In the brain, insulin signalling presents a number of peculiarities compared with peripheral organ insulin function. In the brain, in addition to metabolic and cell growth signalling, insulin and IGF-1 pathways play an important role in synaptic activity (Huang et al., 2003 and 2004; Wang et al., 2000). In particular, this function is supported by an activity-dependent tuning following release from presynaptic terminals upon depolarisation and the ulterior influence on electric transmission at the post-synapse (Wei et al., 1990; Van Der Heide et al., 2005; Cao et al., 2011). This effect has been more widely studied in relation to long-term depression (Insulin-LTD) in the hippocampus, one of the brain regions underlying memory and learning (reviewed in Huang et al., 2009). Thus, the alteration in insulin/IGF-1 signalling with age will have an impact not only on metabolic processes but also on high order brain function. In accordance with this, defects in brain insulin signalling have been associated with the cognitive alterations that accompany brain pathologies of the elderly like Alzheimer's disease (AD) and Parkinson's disease (PD) (Hu et al., 2007; Moloney et al., 2010; Perruolo et al., 2016). However, the precise molecular mechanisms of reduced brain insulin and IGF-1 signalling in the ageing brain are not well known.

Consistent with previous works, we also observed reduced insulin response in the old hippocampus. However, we found an increase in the basal activity of the whole pathway, beginning at the receptor level, affecting both IR and IGF-1R, which is transmitted to these

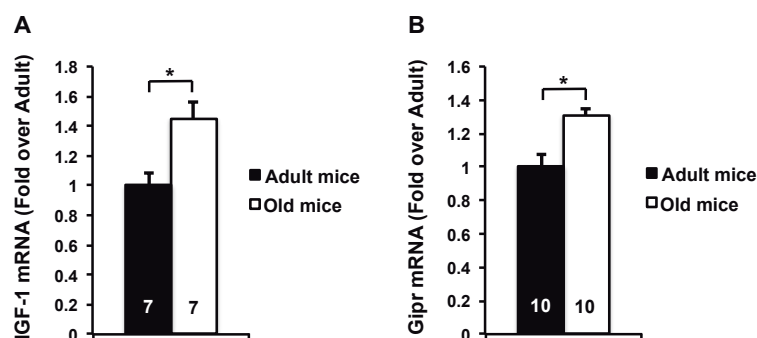
receptors' downstream effectors PI3K/Akt and p70S6K. How a hyperactive pathway results in reduced signalling can be only explained through pathway desensitisation mechanisms, whereby constant activity leads to inhibition. In the case of the insulin and IGF-1 pathway the negative feedback that impedes continuous signalling (in turn avoiding deleterious effects of constitutive activity) is mediated by the induction of phosphorylation at serine residues (Serine 632 and Serine 307) in the IRS-1 scaffolding protein. While other groups have previously described similar events in the context of pathology, in particular in AD (An et al., 2003; Griffin et al., 2005; Talbot et al., 2012), and considered this a negative event, the fact that we observe the same in the normal ageing context, where there is no cell death, suggest that it may serve a beneficial role: to improve survival. In fact, the PI3K/Akt is one of the most conspicuous survival pathways in the brain (Hetman et al., 2000; Chen et al., 2001; Kim et al., 2002; Tschopp et al., 2005; Miyawaki et al., 2009). On the other hand, it is clear that the activation of the PI3K/Akt pathway has negative consequences for synaptic plasticity. Thus, Akt inhibits Gsk3 $\beta$  by phosphorylating this enzyme at the Serine 9 residue, and this inhibition results in reduced AMPARs lateral diffusion and endocytosis and, consequently, impaired LTD (Du et al., 2010; Yagishita et al., 2015). Our results in old hippocampus confirm this negative outcome: together with a higher Akt activity we report inhibited Gsk3 $\beta$  and, more importantly, absence of Insulin-LTD. All these results, added to our demonstration that Insulin-LTD can be rescued when the PI3K/Akt pathway is inhibited, are compatible with the view that an hyperactive insulin/IGF-1 pathway contributes to the mild deficits in memory and cognition that characterise the late stage of life. A "master" determinant of this deficit seems to be the hyperactive PI3K/Akt: it induces the negative feedback on the IRS-1, blocking incoming insulin function (Zick et al., 2001) and inhibits Gsk3 $\beta$  activity and by this means LTD (Peineau et al., 2007). But, as said, the "bad" outcome of PI3K/Akt comes with a "good" counteraction, due to the role of the PI3K/Akt in cell survival. We propose that these "good and bad" actions from the same pathway may reflect a "perform or survive" dilemma of old neurons. Old neurons are characterised by the presence of toxic material accumulated over years that interfere with function and, at the same time, with old and dysfunctional organelles (i.e. mitochondria and the proteasome). Because of these negative conditions function is impaired. Then, a big dilemma of an old neuron arises: reinforce performance or survival? From the data here presented it seems that the answer is an obvious one: survival. This cannot come as a surprise as adaptation for survival is the most conserved mechanism of evolution. The price for survival is, apparently, the reduction in performance.

## **2. On the association between brain insulin defects and peripheral insulin resistance**

Numerous clinical retrospective studies and direct laboratory experimentation support the notion that insulin resistance favours the development of AD signs, by the direct effect of insulin resistance on brain cells (Matsuzaki et al., 2010; Tolppanen et al., 2013). Therefore, a reasonable possibility to explain the reduced insulin signalling in the old brain is that our observations are a simple extension within the brain of whole body insulin resistance. This however does not seem to be the case. The insulin tolerance test performed, that measures insulin sensitivity in peripheral tissues, failed to show significant differences between adult and old mice, of the same age that show the brain insulin signalling defects. Thus the results we obtained using hippocampal fractions seem to reflect a reduction in insulin sensitivity restricted to this brain structure, independently of the peripheral context. This however does not rule out that when peripheral insulin resistance develops it will further impact insulin sensing at the brain level. In any event, our results strongly suggest that hippocampal insulin resistance in the old develops independently from body insulin dyshomeostasis.

## **3. On the mechanism behind brain insulin resistance with age: age-associated cholesterol loss is implicated**

Activation of insulin/IGF-1 signalling pathways in the old brain could be due to different events. One possibility is an increment in peptide/ligand availability to receptors, either because of larger passage from the circulation or because of increased synthesis. In fact, brain cells are able to synthesise both peptides (Bach et al., 1991; Mehran et al., 2012) and both ligands have the capacity to cross the blood-brain barrier. It has been suggested that this double source of inputs allows a signalling compartmentalisation for both peptides, with the brain-external source mainly used in metabolic homeostasis and developmental processes, and the local synthesis responsible for synaptic plasticity modulations (Wei et al., 1990; Fernandez et al., 2012). In support that increased own synthesis might be one of the mechanisms contributing to insulin resistance in the old, we observed higher levels of IGF-1 mRNA in the hippocampus of old mice (Figure D1). Furthermore, we also observed enhanced expression of the insulin signalling facilitator GIP receptor, (Faivre et al., 2011; Paratore et al., 2011). On the contrary, a series of studies have shown that insulin and IGF-1 production becomes reduced with ageing (D'Costa et al., 1995; Frölich et al., 1998; Lee et al., 2014), leaving open the discussion about the role of ligand excess as one of the mechanisms for hippocampal insulin desensitisation with age.



**Figure D1. Insulin/IGF-1 related mRNAs are upregulated in old mice.**

RT-qPCR analysis of IGF-1 peptide (A) and Gpr receptor (B) mRNA transcribed in adult and old mice hippocampus. Data are represented as mean  $\pm$  SEM (Mann-Whitney U-Test for (A) and t-test for (B); \* $p$ <0.05).

Another mechanism that could lead to receptor activation and desensitisation is by age-associated changes in membrane lipid composition, which due to changes in the liquid order balance (rigidity/fluidity) would affect receptors' lateral mobility, clustering and thus autophosphorylation. In this sense, previous works in different laboratories, including ours, have demonstrated the existence of changes in different lipids in the ageing brain (see Colin et al., 2016). Among the lipids that change in the ageing brain, and with a great potential to underlie signalling defects like the ones observed here, cholesterol loss acquires fundamental relevance (reviewed in Martin et al., 2014a and Colin et al., 2016). In support that this may be a cause for the insulin signalling defects, the replenishment of cholesterol to the hippocampus of old mice reduced PI3K/Akt activity, prevented the occurrence of the negative phosphorylation on the IRS-1 protein and restored the functional sensitivity of old hippocampal neurons to exogenous insulin (Insulin-LTD). In further support of a role as "maker" and not simply "marker" of insulin insensitivity, removing cholesterol from the plasma membrane of mature (not old) hippocampal neurons was sufficient to elicit biochemical desensitisation. It is important to notice at this point that the cholesterol loss is likely to affect the level of activity of both insulin and IGF-1 receptors. Both receptors are structurally similar and their activation leads to the downstream activation of similar targets, being the PI3K/Akt node one of the important connections between both signalling factors (Taniguchi et al., 2006). The interrelation of both pathways is an important aspect in insulin-mediated synaptic plasticity, guaranteeing that this type of synaptic plasticity can occur in any place brain region, irrespective of which one, insulin or IGF-1, is made (Wang et al., 2000).

Despite the clear effect that cholesterol loss exerts on insulin signalling in the old brain, one must not forget that: i) cholesterol loss will have a panoply of consequences in addition to the one described here (this laboratory has demonstrated the effect of age-associated cholesterol loss in relation to other survival and plasticity events), including, possibly, glucose uptake and ii) the biological consequences of cholesterol loss with age cannot be dissociated

from the changes in other lipids of the neuronal plasma membrane with age, especially of those that constitute the core of the raft domains: i.e. sphingomyelin and gangliosides. These two important matters should be the subjects of future investigations.

#### ***4. Both insulin and IGF-1 receptors are activated by cholesterol loss, being responsible for (part of) PI3K/Akt high activity in the old hippocampus***

The results described point to cholesterol loss-mediated activation of the PI3K/Akt pathway as the master switch responsible for the insulin desensitisation observed in old mice. However, direct proof that the PI3K/Akt desensitisation came from a cholesterol loss-mediated activation of the insulin or IGF-1 receptor was missing. We confirmed this to be the case by inhibiting the activity of both receptors in neurons with reduced cholesterol with the specific inhibitor Osi-906. This significantly reduced Akt activity and IRS-1 Serine 632 insulin desensitisation mark. It is worth clarifying that IGF-1R signalling inhibition alone did not show a significant reduction effect on any of the two parameters (PI3K/Akt and Serine 632 on IRS-1), suggesting that desensitisation of the pathway is either the consequence of the IR alone or that joint signalling from both receptors is required. The use of IR specific inhibitor will help to clarify this issue. It is also worth pointing out that not all Akt activity was blocked with the IR and IGF-1R inhibitor. We attribute this partial effect to the contribution of other pathways activated by cholesterol loss, like that mediated by the TrkB receptor (Martin et al., 2008).

In addition to cholesterol loss-PI3K/Akt-mediated insulin desensitisation coming from membrane receptors, pathway desensitisation may also be the consequence of the inflammation that occurs in the brain of old subjects (Salminen et al., 2012). Two of the proteins tightly linked with inflammation, Jnk and IKK $\beta$ , have been described to trigger or promote respectively the inhibitory phosphorylations on the IRS-1 protein associated to insulin desensitisation. In addition to the negative phosphorylation on this scaffold protein by the PI3K/Akt/p70S6K cascade (Um et al., 2004), the IRS-1 residue Serine 307 can be negatively regulated by Jnk phosphorylation or by IKK $\beta$  (Aguirre et al., 2000; Yuan et al., 2001). Besides, different inflammatory effectors have been also linked with Serine 307 phosphorylation and insulin desensitisation, as it is the case of p38 (Al-Lahham et al., 2016; Pereira et al., 2016). From these series of observations, the arising conclusion is that hippocampal insulin desensitisation with age is not only the consequence of cholesterol loss on membrane receptors of the tyrosine kinase family (like IR, IGF-1R and TrkB) but also of age associated inflammatory conditions. When we tested this possibility we obtained mixed results: inhibition of Jnk and p38 reduced the negative phosphorylation on Serine 307 but it did not rescue Insulin-LTD. The simplest conclusion would be that despite increase inflammatory inputs leading to higher

negative phosphorylation on IRS-1, the abrogation of this effect is not enough to restore Insulin-LTD. This is however an expected outcome, as lack of Insulin-LTD is the consequence of high PI3K/Akt (via inhibition of Gsk3 $\beta$ ) and reduction of the negative phosphorylation on IRS-1, despite ameliorating insulin's sensitivity, does not reduce PI3K activity. Altogether these data point to the cholesterol loss as the main cause for IR/IGF-1R activation and posterior desensitisation in old mice.

### ***5. Cholesterol loss induces insulin desensitisation through an enhancement of receptors' autophosphorylation***

After we became convinced that plasma membrane cholesterol loss is a key event in the high PI3K/Akt activity in the hippocampus of old mice and that this is in turn a major determinant of insulin desensitisation, we then asked the simple question of how cholesterol loss induces high PI3K/Akt activity. Previous works have demonstrated that the mild membrane cholesterol loss that occurs in the old is not severely deleterious to neurons, probably because the occurrence of major permeability and structural losses might be compensated by, among other mechanisms, changes in the levels of other lipid moieties, including sphingomyelin (Trovò et al., 2011; Colin et al., 2016). This type of lipid re-arrangement of the plasma membrane is also evident in type 2 diabetes mellitus (T2DM) cells (Bryszewska et al. 1986; Watala et al., 1990; Bakan et al., 2006), being especially remarkable the tendency of membranes in diabetics to become more rigid, property that could affect receptors' membrane dynamics and activity (Pilon et al., 2016). In agreement with the possibility that the lipid changes could alter IR/IGF-1R activity directly, works from other laboratories have shown that a reduction in cholesterol levels in adipocytes affects IR signalling (Parpal et al., 2001). Likewise, chronic stimulation of adipocytes with TNF $\alpha$  alters cholesterol/gangliosides proportion leading to a shift in IR location to regions enriched in GM3 gangliosides and affecting post-receptor signalling (Kabayama et al., 2005 and 2007). All these data reflect the importance of membrane composition in IR/IGF-1R signalling. In this sense, our FRET experiments directly prove that cholesterol loss induces a conformational change in the IR/IGF-1R similar to that triggered by ligand (Murakami et al., 1991; Hubbard et al., 1994; Kavran et al., 2014): i.e. approximation of the transmembrane and intracellular domains of the dimer subunits (from there the energy transfer), thus leading to receptor autophosphorylation and consequent activation. Although our experiments were conducted, for practical reasons, in IGF-1R donor and acceptor transfected Hek 293T cells, we consider the increase FRET in these cells as the "proof-of-concept" that a chronic and steady cholesterol loss can directly determine pathway desensitisation by enhancing receptors' activity. Nevertheless, further experiments are required in order to determine if changes in membrane fluidity in neurons from old mice

are behind these receptors high basal activity. In any event, the results here presented pave the way to determine if the mechanism here described for the insulin and IGF-1 receptors is translatable to other types of receptors that also require clustering in cholesterol-rich domains. In relation to this possibility, preliminary results based on the use of a kinase-based screen for 36 receptor tyrosine kinases (RTK), revealed that only five receptors of this family (including IR, IGF-1, VEGF and TrkB) were significantly activated by cholesterol loss. Not only does this rule out broad and unspecific consequences of cholesterol loss but also comes to confirm that different RTKs require different environments for a better signalling (Galbiati et al., 1998; Roepstorff et al., 2002). Thus, although cholesterol loss with age will affect a number of pathways that converge on the PI3K/Akt pathway and thus modulate survival and plasticity by different mechanisms, it appears reasonable to conclude that cholesterol loss with age plays a deterministic role in age-associated insulin deficits by, among other possibilities, directly influencing the receptors' conformation in the plane of the membrane.

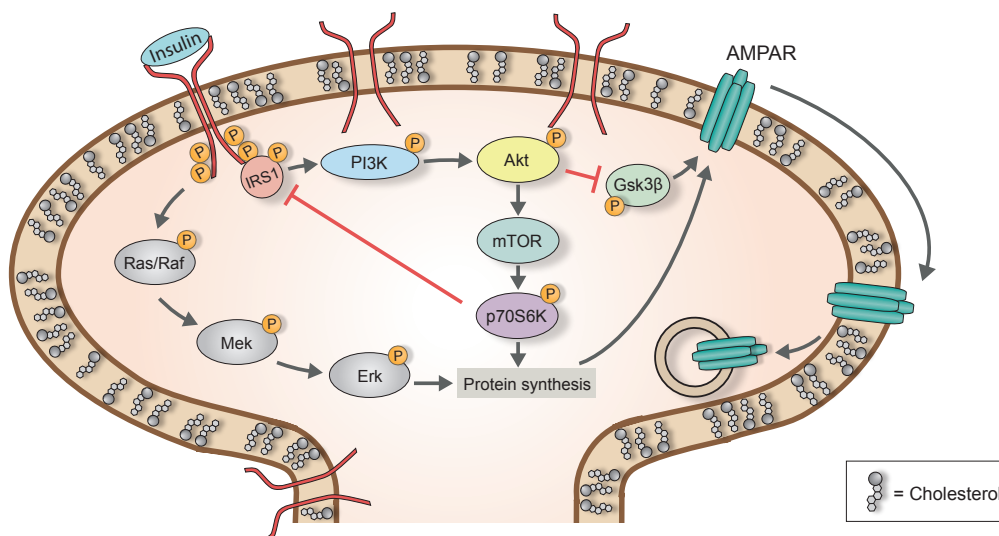
## ***6. Preventing age-associated brain cholesterol loss rescues insulin-signalling deficits***

The cholesterol variations in the brain and the implications for brain health have been subjected to studies for years (Pfrieger et al., 2011; Martin et al., 2014a). Cholesterol alterations correlate with different diseases, some of them of hereditary origin and early appearance (demonsterolosis, Niemann Pick disease type C) and some of unknown origin and late occurrence, like AD. Consequently, numerous laboratories have attempted different types of approaches to rescue diseases associated with such variations. For example, one of the treatments proposed (and in clinical trial) for Niemann Pick type C disease is based on the use of cyclodextrins in order to sequester cholesterol from places with high contents of it. This strategy was seen to stop the progress of the disease in animal models (Davidson et al., 2009). Another strategy was based on the use of LXR receptor agonists, in order to activate cholesterol release from glial cells (see introduction, paragraph 3.1.2). This strategy was shown to ameliorate A $\beta$  clearance and to improve memory in AD mouse models (Donkin et al., 2010). At the same time and due to the vascular deficits associated to AD (Orsucci et al., 2013), the treatment of AD patients with statins for reducing cholesterol levels have been tested. However the results of these trials have to be carefully considered. While some studies reveal positive results and reduction in dementia progression in human (Wong et al., 2013), there are no data to demonstrate that the beneficial effects are due to changes in brain cholesterol content. In fact, it is now proposed that any beneficial effect of statins might be due to either an improvement in overall brain oxygenation due to the blood cholesterol reduction effect of statins or to the anti-inflammatory effect of these compounds or to both.

In contraposition to the observed beneficial effects of statins, other clinical studies revealed no changes or even cognitive impairment under statin treatments, both in mice and humans (Wagstaff et al., 2003; Trompet et al., 2010; Schilling et al., 2014). In this context, the United States Federal Drug Administration raised a warning in 2014 about statins' deleterious effects like confusion, memory loss or cognitive impairment, especially in old people. Considering that age-associated cholesterol loss in the hippocampus has a negative impact on synaptic plasticity (Trovò et al., 2013; Martín et al., 2014b; Palomer et al., 2016b), the last series of clinical results and the FDA warning came as no surprise to us. Thus, in order to attempt to rescue the reduced insulin signalling of the old we treated mice with a drug that inhibits CYP46A1, the enzyme responsible for cholesterol oxidation and removal from brain cells. We induced inhibition of CYP46A1 through the oral administration of Voriconazole, which significantly reduced the levels of activation of the insulin/IGF-1 downstream pathways, including PI3K/Akt and Gsk3 $\beta$  negative phosphorylation, and the inhibitory marks on IRS-1. As importantly, Voriconazole also rescued insulin functional signalling (Insulin-LTD). However, it is not clear that this last effect was through the same mechanism that rescues insulin biochemical signs of desensitisation. The Insulin-LTD rescue effect was observed after 1 hour of incubation with this drug whereas the effects on the biochemical signs were observed after 45 days of treatment. We observed that the chronic treatment rescued cholesterol loss of the old, allowing us to predict that the biochemical rescue is due to a membrane cholesterol restoration. On the other hand, the electrophysiology effects are difficult to see in that same context, as they occur too fast to depend on a membrane cholesterol rescue. Instead, it is possible that the effects observed at the electrophysiology level are the consequence of a reduction in the production of the oxidised cholesterol (24OHC), which was shown to have toxic effects (Kölsch et al., 1999 and 2001). Further electrophysiological experiments in long-term Voriconazole-treated mice would be required to determine if the functional rescue occurs by the cholesterol rescue mechanism.

Considering all the results obtained in this thesis we propose the following model for the hippocampal insulin/IGF-1 signalling progression with age. In adult mice (7-12 month-old) the plasma membrane maintains receptors in a non-activity basal state, which only changes upon ligand binding. Under this condition Insulin-LTD and all other functions of insulin and IGF-1 (Fig. D2) can progress. In the old mice (20-24 month old) mild but steady loss of cholesterol gradually increases basal level of insulin/IGF-1 receptor activation independently from binding to ligand, in the end creating a two-pool IR/IGF-1R receptor environment: insulin sensitive and constitutively active. With advanced age, the constitutive active pool increases. This last event induces, in concert with other pathways activated by the cholesterol loss phenomenon, a most robust activation of the PI3K/Akt pathway that triggers a series of negative events. One of the negative events is the inhibition of insulin/IGF-1 signalling due to the negative feedback on the IRS-1 scaffold, reducing sensitivity to insulin stimuli. Another negative effect is the inhibition of AMPARs internalisation due to Gsk3 $\beta$  inhibition, leading to reduced Insulin-LTD (Fig. D3). On the other hand, all what we have just considered “negative” may in fact be a evolutionary conserved mechanism to assure survival of old neurons: by reducing insulin synaptic plasticity mechanisms through the cholesterol loss-mediated (and other) mechanisms, cells on the one hand make the survival branch of this pathway more robust and, at the same time, reduce a highly energy demanding process such as synaptic transmission. In this context, it appears as if the worsening of performance that we suffer with age is the cost for keeping our neurons alive. We then propose that neurodegenerative diseases of the old like Alzheimer’s, associated or not with T2DM, occur because of defects in the survival mechanisms leading to neuronal death. If this assumption is correct, strategies aimed at improving synaptic function based on cholesterol-replenishment strategies might be deleterious, as they would reduce survival function. These are venues for future studies.

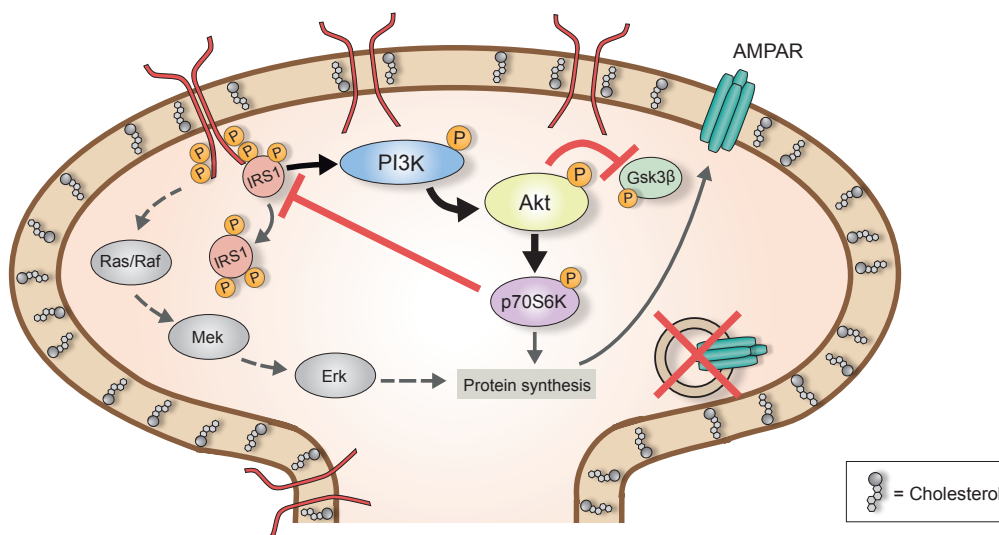
## Adult mice



**Figure D2. Adult mice model for insulin/IGF-1 signalling in the brain.**

The model represents the insulin/IGF-1 signalling pathway in adult mice, where the plasma membrane levels of cholesterol are kept at a normal rate, and IR/IGF-1R are activated by their cognate ligands leading to downstream signalling in two major directions: PI3K/Akt and MAPK/Erk.

## Old mice



**Figure D3. Old mice model for insulin/IGF-1 signalling in the brain.**

The figure represents the insulin/IGF-1 signalling pathway in old mice. Cholesterol levels are reduced in aged mice, possibly (but not exclusively) due to CYP46A1 progressive activation. This situation leads to a higher basal activation of IR and IGF-1R, potentiation of PI3K/Akt pathway leading to IRS-1 negative phosphorylation and consequently insulin/IGF-1 desensitisation and at the same time Gsk3β negative phosphorylation, inhibition of neurotransmitter receptor (AMPA) internalisation and, as consequence, reduced Insulin-LTD.

# *CONCLUSIONS*

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1. The synaptic plasticity event induced by insulin, called insulin-dependent long-term depression (Insulin-LTD), is impaired in the hippocampus of old mice.
2. Hyper-activation of the insulin downstream signalling pathway PI3K/Akt is responsible for the lack of Insulin-LTD in the old hippocampus.
3. Hyper-activation of the insulin downstream signalling pathway PI3K/Akt induces insulin signalling desensitisation through IRS-1 Serine phosphorylation.
4. Hyper-activation of the insulin downstream effector PI3K/Akt prevents Insulin-LTD by Serine 9 phosphorylation on Gsk3 $\beta$ .
5. In addition to the PI3K/Akt signalling node activation other signalling routes, e.g. inflammation, contribute to age-associated insulin desensitisation via the Jnk and p38 MAPK routes.
6. The higher PI3K/Akt activity in the old mice is in part due to high basal activation state of the insulin and the insulin-like growth factor-1 receptors (IR/IGF-1R).
7. The insulin desensitisation observed in old mice hippocampus occurs independently from a general, body, defect in insulin signalling.
8. Age-associated cholesterol loss plays a role in old mice hippocampus IR/IGF-1R desensitisation.
9. Cholesterol loss contributes to pathway desensitisation by producing the conformational changes in IR/IGF-1R required for receptor's autophosphorylation.
10. Prevention of age-associated brain cholesterol loss *in vivo* by the oral administration of a CYP46A1 inhibitor rescues biochemical and functional signs of insulin signalling deficits.

# *CONCLUSIONES*

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1. Los eventos de plasticidad sináptica inducidos por insulina, llamados *long-term depression* (LTD) dependiente de insulina, se encuentran afectados en el hipocampo de ratones envejecidos.
2. La elevada actividad de la vía de PI3K/Akt, relacionada con la insulina, es la responsable de la pérdida de LTD asociada a insulina en el hipocampo de ratones envejecidos.
3. La elevada actividad de la vía asociada a insulina PI3K/Akt causa la pérdida de sensibilidad a insulina a través de la fosforilación en Serinas en IRS-1.
4. La elevada actividad de la vía asociada a insulina PI3K/Akt evita la LTD dependiente de insulina a través de la fosforilación en Serina 9 de Gsk3 $\beta$ .
5. Además de la señalización de PI3K/Akt otras vías, como por ejemplo las inflamatorias, contribuyen a la pérdida de sensibilidad a insulina asociada al envejecimiento a través de las vías de Jnk y p38 MAPKs.
6. La elevada actividad de PI3K/Akt en ratones envejecidos se debe en gran parte a una mayor actividad basal de los receptores de insulina e IGF-1 (IR/IGF-1R).
7. La pérdida de sensibilidad a insulina observada en ratones envejecidos es independiente de un defecto general, en el resto del organismo, de señalización de la insulina.
8. La pérdida de colesterol asociada a la edad juega un papel importante en la reducida sensibilidad de IR/IGF-1R en el hipocampo de ratones envejecidos.
9. La pérdida de colesterol contribuye a la disminución de la sensibilidad de la vía causando los cambios conformacionales en IR/IGF-1R requeridos para la autofosforilación y activación de los receptores.
10. La prevención de la pérdida de colesterol *in vivo* en cerebro, a través de la administración oral de un inhibidor de CYP46A1, rescata los defectos a nivel bioquímico y funcionales de la señalización por insulina.

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