Antioxidants and PPARγ agonists protect the mitochondria of hippocampal neurons from neurodegenerative processes

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Resumen

Las mitocondrias y su función metabólica cambian con el envejecimiento y pueden ser el factor más importante en el desarrollo de diferentes enfermedades neurodegenerativas relacionadas con la edad, incluida la enfermedad de Alzheimer (EA). En esta condición patológica, las mitocondrias muestran un potencial de membrana reducido, aumento de la permeabilidad, dishomeóstasis del calcio con una producción excesiva de especies reactivas de oxigeno (ROS), pudiendo producir un daño importante en las proteínas, lípidos y ácidos nucleicos de la células.

En la EA, la sobreproducción de la proteína precursora del amiloide (APP) y del péptido β-amiloide (Aβ) por el procesamiento proteolítico de la APP, junto a los ovillos neurofibrilares producto de la hiperfosforilación de la proteína Tau, pueden afectar el equilibrio dinámico mitocondrial (fusión/fisión), aumentando la fisión. En particular, el Aβ es capaz de interactuar con las membranas y proteínas mitocondriales, contribuyendo a la fisiopatología de la neurodegeneración.

En esta tesis doctoral nos hemos centrado en tratamientos dirigidos a mejorar el metabolismo de las mitocondrias con estrategias como es el uso de antioxidantes que regulen la producción de ROS en las neuronas, pero que también contribuyan a la biogénesis mitocondrial. El uso de la quercetina un antioxidante presente normalmente en los frutos rojos y plantas del género *Allium*, con bajo nivel de actividad antioxidante se ha visualizado como una terapia eficaz, porque es capaz de proteger a la neuronas del daño producido tanto por el estrés oxidativo como por el péptido Aβ, pero además puede activar vías de señalización relacionadas con la defensa celular antioxidante, como es la activación del factor de transcripcion erytheroid-derived-2-like 2 (Nrf2), resultando en el aumento de los niveles de catalasa y peroxidasa en la célula.

Por otra parte, agonistas de los receptores de activadores de proliferación peroxisomal del tipo gamma (RAPPgamma), usados en la actualidad en el tratamiento de la diabetes tipo 2 han demostrado también una actividad anti-inflamatoria. Estas moléculas pueden ser utilizados para inducir la biogénesis mitocondrial, reestablecer la homeostasis del calcio y proteger a las neuronas del daño oxidativo. Es en este sentido que la búsqueda de moléculas que ayuden a restablecer la homeostasis celular y proteger del daño oxidativo a las neuronas son importantes para el tratamiento y/o prevención de las enfermedades neurodegenerativas.

Abstract

Mitochondria and their metabolic function change with aging and these events may be the most important factor in the development of different neurodegenerative diseases related to age, including Alzheimer's disease (AD). In this pathological condition, the mitochondria show a reduced membrane potential, increased permeability, dishomeostasis of calcium with an excessive production of reactive oxygen species (ROS), and may cause significant damage to the proteins, lipids and nucleic acids of the cells.

In AD, the overproduction of amyloid precursor protein (APP) and β-amyloid peptide (Aβ) by the proteolytic processing of APP, together with the neurofibrillary tangles resulting from the hyperphosphorylation of the Tau protein, can affect the mitochondrial dynamic equilibrium (fusion/fission), increasing fission. In particular, Aβ is able to interact with mitochondrial membranes and proteins, contributing to the pathophysiology of neurodegeneration.

In this doctoral thesis, we have focused on treatments aimed at improving the metabolism of mitochondria by strategies such as the use of antioxidants that regulate ROS production in neurons, but also contribute to mitochondrial biogenesis. The use of quercetin, an antioxidant normally present in red fruits and plants of the genus *Allium*, with a low level of antioxidant activity has been seen as an effective therapy, because it is able to protect the neurons from the damage caused by both oxidative stress and it can also activate signaling pathways related to antioxidant cellular defense, such as the activation of the transcription factor erytheroid-derived-2-like 2 (Nrf2), resulting in increased levels of catalase and peroxidase in the cell.

On the other hand, agonists of peroxisomal proliferation activator receptors of the gamma type (PPARgamma), currently used in the treatment of type 2 diabetes have also demonstrated an anti-inflammatory activity. These molecules can be used to induce mitochondrial biogenesis, re-establish calcium homeostasis and protect neurons from oxidative damage. It is in this sense that the search for molecules that help to restore cellular homeostasis and protect from oxidative damage to neurons are important for the treatment and/or prevention of neurodegenerative diseases.

Prologue

The brain is particularly dependent on high glucose and energy levels for proper neuronal function; therefore, it is rich in mitochondria. Mitochondria are essential organelles involved in ATP production, fatty acid-β-oxidation, oxygen metabolism, iron-sulfur clusters, survival, apoptosis, and they are also involved in the maintenance of calcium homeostasis and signaling.

The metabolic alterations of mitochondria are responsible for the induction of dysfunction with a severely detrimental impact on cell physiology mainly via bioenergetics impairment, calcium dyshomeostasis and apoptosis deregulation. Mitochondria are also responsible for ROS production, which occurs when unpaired electrons escape from the ETC and react with molecular oxygen. The rate of ROS production depends mostly on the mitochondrial membrane potential ($\Delta\psi m$), the hyperpolarization and dissipation of the mitochondrial membrane potential leads the mitochondrial membrane could lead to an increase in ROS production. In consequence, several neurodegenerative disorders age-related, have been related to impaired mitochondrial function, including Alzheimer disease (AD).

The main aim of the present doctoral thesis is to study mitochondrial dynamics in AD. In this sense, we have studied the antioxidant and protective activities of a flavonoid, quercetin, in cultured hippocampal neurons and their effect on mitochondria undergoing oxidative stress and Aβ-induced degeneration. On the other hand, we have also studied the effects of the activation of peroxisomal proliferation activator receptors of the gamma type (PPARγ with a selective peroxisome proliferator activated receptor modulator agonists (SPPARM) named INT131 on cultured hippocampal neurons challenged with Aβ. We used the INT131 and rosiglitazone and analyzed their protective roles in the mitochondria.

Furthermore, the relevance of mitochondrial dysfunction in the context of neurodegeneration was demonstrated according to the mitochondrial hypothesis of AD. Hence, we consider that preventing or rescuing mitochondrial functionality is a central goal for improving the outcome of neurodegenerative disorders.

Abbreviations

AAMI, age-associated memory impairment**; ABAC1**, ATP-binding cassette transporter; **Aβ**, β-amyloid species; **Aβo**, Aβ oligomers; **AChE**, acetylcholinesterase; **AGES**, advanced glycation end products**; AICD,** The amyloid precursor protein intracellular domain**; AMPK**, AMP-activated protein kinase; **AP-1**, activator protein 1; **APP**, amyloid precursor protein; **APOE**, Apolipoprotein E; **ARE**, antioxidant response element; **ASK1**, apoptosis signal-regulating kinase; **ATP**, adenosine triphosphate; **BACE**, Beta-site Amyloid precursor protein Cleaving Enzyme 1; **BBB**, blood brain barriers; **bcl-2,** B-cell lymphoma-2; **bad**, Bcl-2-associated death promoter; **bax**, BCL2 associated X protein; **CaM**, Calmodulin; **CAMKII**, Calmodulin kinase II; **CAT**, choline acetyltransferase; **CDR**, cysteine-rich domains; **ChEIs**, cholinesterase inhibitors; **CNS**, central nervous system; **CTF,** alpha- or beta-secretase-cleaved COOH-terminal fragments of APP; **CUS**, chronic unpredicted stress;**CTFα or CTFβ**, carboxyl-terminal fragments; **COX-2**, cyclooxygenase-2; **Dkk1,** Dickkopf 1; **DRP1**, dynamin-like protein 1 protein; **Dvl**, Disheveled; **EPSC**, excitatory postsynaptic current; **E-cadherin**, E-calcium dependent adhesion; **ephrin**, Eph receptor-interacting protein; **ER**, reticulum endoplasmic; **ETC**, electron transport chain; **eIF2α**, eukaryotic translation initiation factor 2α ; **FADH**, flavin adenine dinucleotide reduced form; **fAD**, familiar autosomal form of AD; **fEPSP**, field excitatory postsynaptic potential; **FDG-PST**, Fluorodeoxyglucose [^{18}F] positron emission tomography; **Foxos**, forkhead box O; **FZ**, Frizzled; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **GSH**, glutathione; **HIF-1**α, hypoxia-inducible factor 1 α; **γ-GCL**, γ -glutamyl cysteine ligase; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **GLP-1**, glucagon-like peptide-1 receptor; **GPX-3**, plasma glutathione peroxidase; **GST**, glutathione S-transferase; **γ-GCL**, γ-glutamyl cysteine ligase; **H_2O_2**, hydrogen peroxide; **HDL**, high density lipoproteins; **hNSCs**, human neural stem cells; **HO-1**, heme oxygenase-1; **HSF-1**, Heat shock factor-1; **iNOS**, induced nitric oxide synthase; **IICR**, IP3-induced Ca2+ release; **IP_3R**, Inositol 1,4,5-triphosphate receptors; **JNK**, Jun N-terminal kinase**; KEAP1**; Kelch-like ECH-associated protein1; **KGDHC**, alpha-ketoglutarate dehydrogenase complex; **LXRs**, liver X receptors; **LDH**, lactate dehydrogenase; **LDLR**, low density lipoprotein receptor; **LRP1**, lipoprotein receptor-related protein 1; **MCI**, mild cognition impairment; **MDH,** malate dehydrogenase; **MMP**, mitochondria membrane potential; **MMTV**, mouse mammary tumor virus; **MnSOD**, manganese superoxide dismutase; **mPTP**, mitochondrial permeability

transition pore; **mtDNA**, mitochondrial DNA; **NCX**, Na^+/Ca^{2+} exchanger; **NO**, Nitric Oxide; **nNOS**, neuronal oxide synthase; **NFTs**, neurofibrillary tangles; **NMDAR**, N-methyl-D-aspartate receptor; **NADH**, nicotinamide adenine dinucleotide reducted form; **NQO-1**, NAD(P)H quinone oxidoreductase-1; **NF-κB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **Nrf,** Nuclear Respiratory Factor; **Nrf2**, nuclear factor erytheroid-derived-2-like 2; **NKCC1**, Na+-K+-2Cl- cotransporter 1; **O_2**, molecular oxygen; **OPA1**, optic atrophy protein 1; **ONOO−**, peroxynitrite; **OS**, oxidative stress; **$O_2^{\cdot-}$** , superoxide anion; **$^{\cdot}$OH**, hydroxyl radical; **$^1O_2$**, singlet oxygen; **oxphos**, oxidative phosphorylation; **PKB/AKT**, Protein kinase B; **PPARγ**, peroxisome proliferator-activated receptor-γ; **PPREs,** PPAR response elements; **redox**, reduction-oxidation; **sAβPPα**, α-secretase; **sAβPPβ**, β-secretase; sAD, sporadic AD; **PDHC**, pyruvate dehydrogenase complex; **PEN2**, presenilin enhancer 2; **PM-Ca-ATPase**, plasmatic membrane Ca-ATPase; **PSEN1**, presenilin 1; **PSEN2**, presenilin 2; **PKC**, Protein Kinase C; **PPAR**, peroxisome proliferator-activated receptor; **RAGE**, receptor for advanced glycation end products; **ROS**, reactive oxygen species; **RO$^{\cdot}$**,alkoxyl radicals; **ROO$^{\cdot}$**, peroxyl radicals; **RNS**, reactive nitrogen species; **RXR**, retinoid X receptor; **RyR**, ryanodine receptor; **SD**, succinate dehydrogenase; **SERCA**, sarco/endoplasmic reticulum Ca2+-ATPase; **Sirt1**, Silent Information Regulator 2 Homologue 1; **SOD,** superoxide dismutase; **SPPARM,** selective peroxisome proliferator-activated receptor modulator agonists; **STAT-6**, signal transducer and activator of trancription-6; **TBARS**, Thiobarbituric acid reactive substances; **TFAM.** Mitochondrial transcription factor A**;** **TREM2**, Triggering receptor expressed on myeloid cells 2; **T2DM**, Type 2 diabetes mellitus; **T3DM**, Type 3 diabetes mellitus; **VDAC**, voltage-dependent anion channel; **UCP2**, uncoupling protein 2; **UGT1A1**, uridine diphosphate (UDP) glucuronosyl transferase.

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I. INTRODUCTION

The brain is particularly dependent on high energy levels for proper neuronal function; therefore, it is rich in mitochondria. Mitochondria are the cellular organelle involved in the production of adenosine triphosphate (ATP) through the electron transport chain (ETC), but they also have other key functions, such as neurotransmitter release, calcium homeostasis and signaling. They are also involved in deleterious effects, such as reactive oxygen species (ROS) production and apoptosis (Eckmann et al, 2013).

Mitochondria are also responsible for ROS production, which occurs when unpaired electrons escape from the ETC and react with molecular oxygen, generating ROS. The rate of ROS production depends mostly on the mitochondrial membrane potential ($\Delta\psi m$). Therefore, the dissipation of the mitochondrial membrane potential leads to an increase in ROS generation when respiration is inhibited. On the other hand, the hyperpolarization of the mitochondrial membrane could lead to an increase in ROS production (Angelova and Abramov, 2018) (**Fig. 1**).

ROS can be classified into two groups depending on their structure. There are molecules with an unpaired electron (group 1) and those able to generate molecules with an unpaired electron (group 2). Group 1 is composed of superoxide ($O^{\cdot -}_2$), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), hydroxyl ($OH^{\cdot}$) and hydro- peroxyl ($HO^{\cdot}_2$) radicals. Group 2 includes hydrogen peroxide (H_2O_2), organic peroxides (ROOH), aldehydes (HCOR), hydrochlorous acid (HOCL), nitric oxide (NO), ozone (O_3) and singlet oxygen ($^1O_2$). In particular, NO a product of the ubiquitous nitric oxide synthase (NOS), can react with other ROS to generate reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$) (**Fig. 2**)**.** ROS and RNS are highly reactive and can oxidize nucleic acids, proteins, and lipids (Navarro and Boveris, 2007), which have been shown to be involved in the etiology and progression of several chronic pathologies, such as neurodegenerative diseases (Steinert et al, 2010; di Domenico et al, 2017). ROS also have an important role in intracellular signaling at both the physiological and pathophysiological levels; therefore, the tight maintenance of the physiological ROS levels in the brain is critical for normal neuronal functions, but increased mitochondrial activity without the appropriate control

mechanisms counteracting ROS scavengers can increase the ROS levels, thereby altering the intracellular metabolism and signaling (Balaban et al, 2005).

Mitochondrial dysfunction causes increased ROS production, impaired energy supply, Ca^{2+} dyshomeostasis, reduced glucose metabolism, opening of the mitochondrial permeability transition pore (mPTP) and apoptosis. All these events can contribute to the progressive decline of long-lived neurons, with dramatic cellular consequences linked to aging and several neurological disorders in humans (Onyango et al, 2010). To ensure their physiological functions and maintain their health, the number and morphology of mitochondria are tightly controlled and this control is achieved through the mitochondrial dynamic processes of fusion and fission (McBride et al, 2006).

1.1. Aging

Aging is a systemic process that affects all organs. It is characterized by alterations in cellular functions and the accumulation of diverse deleterious products, which constitute the major risk factor for age-related diseases (Wei and Lee, 2002).

The brain has attracted the attention of gerontologists and researchers because of the increased incidence of neurodegenerative diseases and dementia in people of advanced age. The decrease in cognitive abilities requires alterations in brain structure, loss of synapses and metabolic changes (Raz and Daugherty, 2017). Cognitive traits decline in parallel with aging and are defined as "age-associated memory impairment" (AAMI), which is applied to elderly individuals with minor cognitive impairment, no dementia, and cognitive scores below those of young adults but without any recognized pathology (Crook et al, 1987).

Working memory, a component of executive function, and processing speed decline with age; however, semantic knowledge and autobiographical memory tend to remain intact (Sawa et al, 2009). In this regard, cognitive ability, which is relatively stable in non-demented aged individuals, is localized in the cortex and hippocampus, both of which show age-related changes.

In fact, studies performed with functional imaging to evaluate brain activity show a direct correlation between advanced age and prefrontal cortical atrophy, while hippocampal volumes decrease. These changes implicate damage accumulation and cellular function decline (Kravitz et al, 2012; Hamezah et al, 2017). Neural activity also becomes less localized in some brain regions,

in addition to being less integrated, particularly in the prefrontal cortex, in response to executive level tasks. These observations suggest that neurons themselves and neuronal networks are significantly altered during aging even in the absence of a disease (Bishop et al, 2010; Maillet and Rajah, 2103).

1.1.1. Oxidative stress in aging

Six decades ago, it was proposed that aging could be the expression of cumulative damage inflicted by ROS to the cellular machinery, also known as the "free radical theory" (Harman, 1956). This theory postulates that aging is the result of the high production of ROS and the accumulation of cellular damage due to the deleterious effects of ROS on biomolecules, including nucleic acids, it is this phenomenon where the brain is particularly susceptible to the damaging effects of ROS. Aging process is universal, intrinsic, progressive, irreversible and occurs during the lifespan. (Harman, 1956). The discovery of superoxide dismutase (SOD) and the demonstration that this enzyme *in vivo* catalyzes the dismutation; or partitioning; of the superoxide radical into either ordinary molecular oxygen (O_2) or H_2O_2 gave credibility to the free radical theory because there are specific intracellular mechanisms to detoxify ROS (Harman, 1956; Reiter, 1995; Bokov et al, 2004; Muller et al, 2007).

The free radical theory provides a relevant role to the mitochondria because these organelles generate the majority of ROS within cells, becoming the source of origin and the target of ROS (Sastre et al, 2000). In fact, mitochondrial DNA (mtDNA) deletions and mutations are induced by oxidative stress (OS), damage that accumulates with aging (Wang et al, 2013; Shokolenko et al, 2014).

ROS are produced in multiple places in the mitochondrion, but complexes I and II are the most common locations of ROS production during aging. However, respiratory chain complex I is considered the major source of ROS production, where $O_2^{\bullet-}$ production by the ETC may be regulated by a negative feedback loop. $O_2^{\bullet-}$ produced by the mitochondrial respiratory chain inactivates aconitase. Aconitase is an enzyme with an iron-sulfur cluster that catalyses the stereo-specific isomerization of citrate to isocitrate, and that iron sulfur cluster is highly sensitive to oxidation by $O_2^{\bullet-}$. Inactivation of this enzyme suppresses the Krebs cycle and reduces the supply to the respiratory chain of the reduced form of nicotinamide adenine dinucleotide (NADH) and

the reduced form of flavin adenine dinucleotide (FADH) (Gardner, 2002; Adam-Vizi, 2005). Moreover, in metabolic disorders, there is an increase in free fatty acids, which enhances the serine phosphorylation of insulin receptor substrate 1 (IRS-1) and IRS-2 and impairs insulin signaling in the liver. It results in the activation of NADH phosphate oxidase in the plasma membrane, resulting in ROS formation and OS (Pereira et al, 2014).

Mitochondrial dysfunction is an early pathological event in aging in which the activity of complexes I, III, and IV is decreased (Miquel et al, 1980; Trifunovic & Larsson, 2008). According to the "mitochondrial theory of aging", the accumulation of mutations in mtDNA due to continuous exposure to ROS is a key factor in the decrease of energetic metabolism that characterizes aging. Several studies have been conducted with mtRNA to understand the role of abnormalities in mitochondrial ETC and in the pathogenesis of Alzheimer's disease (AD). In fact, the expression of mitochondrial-encoded genes for complex I is down-regulated in early and late AD brains, while genes for complexes III and IV show increased mtRNA expression, suggesting an increased energy requirement (Manczak et al. 2004).

Recent data suggest that other organelles, such as the reticulum endoplasmic (ER) and peroxisomes, may have higher ROS production during aging (Brown and Borutaite, 2012; Schulz et al, 2014). On the other hand, the cell membrane of neurons and others cell in the brain are composed of phospholipids rich in polyunsaturated fatty acids whose double bonds lead to hydrogen ion removal and increased lipid peroxidation, one of the most prominent features of the degenerative changes in AD (Söderberg et al, 1991; Prasad et al, 1998).

1.1.2. Molecular alterations in aging

Post-translational modifications and structural changes to proteins are key events in aging and these modifications could produce or accelerate the formation of protein aggregates that can be toxic or affect their functions by reducing cellular activity and impairing the ability to resist physiological and external stressors (McEwen and Stellar, 1993). These molecular modifications to biomolecules that occur during aging and especially in AD are due to oxidative stress, nitrotyrosination and protein glycation.

1.1.2.1. Protein oxidation

Reduction-oxidation (redox) activity controls cellular energetics, mitochondrial dysfunction, epigenetics, immune defense and reproduction. It allows a high flexibility and adaptability to the environment and challenges during lifespan (Squier, 2001; Go and Jones, 2017). OS is caused by a general progressive imbalance between the generation of intracellular ROS, mainly produced in mitochondria ($O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet -}$) and the pathways involved in antioxidant defense, such as SOD, glutathione peroxidases and catalase, to repair the resulting oxidative damage (**Fig. 3**). Low energy reserves and high stress levels underlie the selective vulnerability of neurons to OS (Wang et al, 2009). This imbalance is characteristic of neuropathological changes, and these processes ultimately produce death of the mitochondrion, which is particularly vulnerable to oxidative damage (Muller et al, 2007; Shokolenko et al, 2014).

Mitochondria create a critical redox interface between an organism, metabolic and structural systems operating as truly functional networks to support genome adaptation to environmental resource availability (Münzel and Daiber, 2017). Alterations in the normal redox state of tissues or cells can cause toxic effects on proteins, lipids, and DNA. Cellular proteins are highly susceptible to oxidative damage, which inevitably affects their secondary and tertiary structure, resulting in their irreversible modification and consequently impairing their functions. These modifications include dissociation of subunits, protein misfolding and exposure of hydrophobic residues, aggregation and backbone fragmentation. Many of the cerebral enzymes are modified oxidatively with the consequent decrease in their activity; these alterations in the activity can be explained based on protein structure, which expose more hydrophobic sites to the aqueous environment, leading to subsequent changes in conformation and aggregation. These structural modifications yield significant formation of misfolded and aggregated proteins in a time-dependent manner (Berlett and Stadtman, 1997; Dean et al, 1997; Stadtman, 2006; Zhong et al, 2013). In this regard, slightly more than 20% of the newly synthesized proteins are rapidly degraded by the proteasome. It has been observed that reductions in the protein degradation rate in senescent animals will increase protein aggregates (Wickner et al, 1999). Consequently, to minimize the oxidation of intracellular proteins, it is expected that the oxidative environment inside the cell could be decreased, and the degradation in the activated proteasome is expected to

restore cellular homeostasis, preventing the accumulation of misfolded proteins (Turner and Varshavsky, 2000)**.**

Some proteins are selectively oxidized in a reversible manner, such as Calmodulin (CaM), calcium pumps, sarco/endoplasmic reticulum Ca2+-ATPase, or SR Ca2+-ATPase (SERCA) and calcium channels (Viner et al, 1999; Squier, 2001; Park and Reuter-Lorenz, 2009). This process is a physiological way to regulate protein activity, but an imbalance will trigger dramatic metabolic effects. In particular CaM, a protein in rat brain with a relatively long half-life, shows a progressive age-dependent decline in its ability to bind to the plasma membrane Ca-ATPase (PM-Ca-ATPase) when modified by ROS (Ferrington et al, 1997). In cerebral CaM, the progressive age-dependent oxidative modification of multiple methionines has been observed, resulting in a loss of function regarding the activation of the PM-Ca-ATPase. These results are reproducible *in vitro*; thus, exposure of CaM to H_2O_2 results in a similar pattern of oxidative modification of the methionines residues to that CaM isolated from senescent brain (Ferrington et al, 1997). CaM turnover in cells is tightly regulated to maintain constant amounts of this protein with normal function following calcium activation; these latter results indicate that the oxidative modification of CaM may have profound effects on neuronal function (Squier and Bigelow; 2000; Squier, 2001).

1.1.2.2. Protein nitration

The cascade of ROS production is initiated by $O_2^{\cdot -}$ derived from the mitochondrial electron transport chain and the membrane-associated NAD(P)H oxidase complex. On the other hand, NO is produced by nNOS, which is constitutively expressed in neurons and plays an important role in the brain, where it functions as a signaling molecule in various cellular processes (Wang and Michaelis, 2010). Peroxynitrite (ONOO-) can be formed *in vivo* when NO and $O_2^{\cdot -}$ react. Then, a nitro group of peroxynitrite covalently binds to the aromatic ring of tyrosine residues in a pathological irreversible process termed nitrotyrosylation (Ischiropoulos, 2003) (**Fig. 2**). It has been reported that the processing and secretion of Aβ is enhanced in *in vitro* models correlating with the nitrotyrosination of presenilin 1 (PSEN1), the catalytic subunit of γ-secretase that has been reported to be particularly active in aging and AD (Ill-Raga et al, 2010; Guix et al, 2012; Yuan et al, 2012). Moreover, nitrotyrosination of Aβ favors the stabilization of

highly toxic Aβ oligomers (Aβo) and inhibits the formation of Aβ fibrils. Nitrated Aβo are particularly toxic to neurons via their action on N-methyl-D-aspartate receptor (NMDAR), with sustained elevated calcium levels triggering excitotoxicity, a characteristic event in AD (Guivernau et al, 2016).

1.1.2.3. Protein glycation

Proteins can suffer modifications by pathologically binding sugars in a process termed glycation. Protein glycation is due to the formation of Amadori products by a non-enzymatic reaction generating advanced glycation end products (AGEs). AGEs cause cellular and molecular events, including neuronal apoptosis (**Fig. 4**). It has been reported that protein glycation increases the activity of BCL2 associated X protein (bax) and Bcl-2-associated death promoter (bad) in neurons, decreases the mitochondrial membrane potential and decreases the activity of B-cell lymphoma-2 (bcl-2) (Tajes et al., 2014).

1.1.2.4. Protein aggregation

The close relationship between protein aggregation and neurodegenerative diseases has focused research on the pathological folding of proteins. Protein aggregates have been shown in normal aging in species from nematodes to humans (David et al., 2010; Ayyadevara et al, 2016).

These modifications strongly promote aggregation and may be able to increase with age (Ayyadevara et al, 2015). The oxidation levels of neurons can be decreased if protein repair and degradative systems are able to act on oxidized proteins and restore cellular function by eliminating damaged or partially unfolded proteins (Bohlooli et al, 2016). It has been proposed that partially unfolded tertiary structures that readily form aggregates are likely oxidized/nitrated/glycated, contributing to the formation of deposits of insoluble fibrils (Koo et al, 1999). Protein aggregation may occur if protein repair and degradative systems are unable to act on oxidized proteins to restore cellular function (Bohlooli et al, 2016).

1.1.2.5. Calcium homeostasis and signaling

Ionic calcium is one of the major intracellular signaling molecules in eukaryotic cells. It works as a modulator of intracellular metabolism rapidly and coordinately with a wide range of cellular pathways. The energy balance and maintenance of the intracellular Ca^{2+} gradient are necessary for signaling, especially in neurons. It is critical to manage Ca^{2+}-dependent mechanisms maintaining the concentration gradients across the plasma membrane by SERCA and the Na^{+}/Ca^{2+} exchanger (NCX) an antiporter membrane protein that removes calcium from cells. It has been demonstrated that the ability of old neurons to handle Ca^{2+} load/overload is diminished, these changes lead to an increase in the levels of Ca^{2+} and to a substantial prolongation of stimulus-evoked Ca^{2+} signals, which may explain the higher vulnerability of aged neurons to excess calcium and its effects on neurotransmission release and neuronal plasticity (Kirischuk and Verkhratsky, 1996; Ruat and Traiffort, 2013; Takeuchi et al, 2015).

Cytosolic Ca^{2+} is regulated mainly by Inositol 1,4,5-triphosphate receptors (IP_3Rs) located in the ER. This activity shows a typical biphasic pattern with a flow from a region of low Ca^{2+} concentration toward a region with a higher, inhibitory concentration, allowing the cells to respond rapidly to external stimuli via fast and localized increases in cytosolic Ca^{2+} (Verkhratsky and Shmigol, 1996; Shah et al, 2015; Takeuchi et al, 2015). Other Ca^{2+} channels are also located in the ER, such as the ryanodine receptor (RyR), which is responsible for the release of Ca^{2+} from intracellular stores towards the cytoplasm (**Fig. 5**).

On the other hand, in the nerve terminals, the voltage-gated Ca^{2+} channels function in the release of neurotransmitters. Ca^{2+} from intracellular stores regulates spontaneous release at excitatory and inhibitory terminals when specific agonists stimulate their receptors. The molecular machinery in neurons that triggers spontaneous vesicle fusion may differ from the machinery underlying evoked release and may be one of the sources of heterogeneity in release mechanisms leading to a wide spectrum of Ca^{2+} signals (Williams and Smith, 2017). These Ca^{2+} signals activate proteins such as kinases and phosphatases, modulate the growth of the axon, control long-term neuronal differentiation, and play an important role in the basal stages of synaptogenesis, synaptic plasticity and redox state in neurons (Paula-Lima et al, 2014; Faltinova et al, 2017). Ca^{2+} is a key regulator of synaptic plasticity, and therefore, it is easy to see how dysregulation of Ca^{2+} could lead to cognitive abnormalities, playing a central role in

excitotoxicity by the consequent activation of Ca^{2+}-dependent proteases (caspases and calpains) and also the breakdown of cytoskeletal proteins (Tremper-Wells and Vallano, 2005).

1.2. Alzheimer's Disease

AD is a progressive, age-associated neurodegenerative disorder characterized by progressive neuronal loss, cognitive dysfunction and memory loss. It is a complex, progressive and irreversible neurodegenerative disease and is the most common form of dementia in the elderly (Selkoe et al, 2012; Bloom, 2014). The neuropathology of AD includes the presence of extracellular amyloid plaques, by accumulation of extracellular amyloid (Aβ), in the parenchyma as diffuse, focal or stellate deposits and intraneuronal neurofibrillary tangles (NFTs) constituted mainly by hyperphosphorylated tau protein. NFTs are in the neuronal body and dendrites, and their progression is stepwise and stereotyped from the entorhinal cortex through the hippocampus and therefore to the cortex. Tau aggregation drives local neurodegeneration, whereas the relationships between Aβ and neurodegeneration are not region specific and may be mediated by a complex interaction between Aβ and tau, which leads to the loss of neurons and synapses correlating with the clinical symptoms (Hardy and Higgins, 1992; Iaccarino et al, 2017) (**Fig. 6**).

On the other hand, microglias have important functions during development and contribute to maintaining cerebral homeostasis, but they can also trigger neuroinflammatory processes contributing to the pathogenesis of neurodegenerative diseases such as AD (Walter et al, 2017).

Patients with senile dementia were traditionally excluded from AD, despite its similarities, because senile dementia was generally considered an age-associated phenomenon and not a true disease. However, because Aβ plaques and NFTs are frequently present in the brain of patients with senile dementia, researchers eventually expanded the definition of AD to include those with senile dementia, plaques and tangles (Grundke-Iqbal et al, 1986; Serrano-Pozo et al, 2011; Selkoe et al, 2012). Macroscopic brain changes in AD include progressive brain atrophy, principally in the hippocampal formation, it has been shown that hippocampal atrophy occurs early in AD. Measuring the rates of baseline and hippocampal atrophy allows for the discrimination of mild cognition impairment (MCI) from cognitively normal subjects. On the other way, whole-brain volume is the best metric for discriminating between AD and MCI, and

whole-brain atrophy is readily evident in later-stage AD (Henneman et al, 2009). Early symptoms typically include difficulty remembering names and recent events, apathy, and depression, while later symptoms include worsening memory, impaired judgment, disorientation, confusion, behavioral changes, and difficulty speaking, swallowing and walking. However, the most important consequence of Aβ is synaptic dysfunction, yielding cognitive impairment and memory loss (Duyckaerts et al, 2009; Bloom, 2014).

1.2.1. Familial and Sporadic AD

The early-onset familial autosomal form of AD (fAD) accounts for approximately 1-2% of cases, while late-onset or sporadic AD (sAD) represents 98-99% of all cases worldwide. Several dominant familial genes cause fAD. Mutations in the amyloid precursor protein (APP) gene result in increased processing of this protein through the amyloidogenic pathway, but this gene accounts for a small percentage of AD cases (Haass et al, 1994; Acx et al, 2013). FAD includes genes involved in APP processing, such as PSEN1-2, which are components of the γ-secretase, a protein complex required for Aβ production. The γ-secretase consists of 4 essential subunits: PSEN1 is the catalytic subunit, acting as an aspartyl protease, nicastrin, anterior pharynx defective (APH-1), and presenilin enhancer 2 (PEN2) that assemble in a 1:1:1:1 stoichiometry. The γ-secretase cleaves APP, notch-1, E-calcium dependent adhesion protein (E-cadherin), Eph receptor-interacting protein (ephrin) B2, triggering receptor expressed on myeloid cells 2 (TREM2) and type I membrane proteins with very small ectodomains (Marambaud et al, 2002; Laurent et al. 2015). Because of the involvement of γ-secretase in the processing of APP to produce Aβ, research has focused on the function of this protease in neurons; however, the ubiquitous expression of this protease in various cell types and organs, as well as its large number of substrates, make it difficult to study the specific role of this enzyme in AD. There is also evidence that γ-secretase regulates the migration, proliferation and phagocytosis of microglia (Farfara et al, 2011).

On the other hand, sAD appears after 65 years of age, yielding severe behavioral and cognitive impairments. Clinical studies carried out with patients to evaluate the time between the first symptoms and the diagnosis of the disease have produced two types of results. First, there were more cases in individuals with a higher frequency of histories of dementia and personal

histories of psychiatric disorders. Second, there were no differences between early-onset and late-onset AD from a clinical and/or neuropsychological perspective (Vilalta-Franch et al, 2007). Other studies demonstrated that compared with sAD patients, fAD patients with two APOE ε4 alleles showed a faster decline on cognitive tests (Wattmo et al, 2017).

Regarding the combined treatment with acetylcholinesterase inhibitors (ChEIs) and NMDAR antagonists, the main therapies for mild to moderate AD, no clinical differences were reported between sAD and fAD patients. This study was carried out by analyzing global cognitive function and specific neurocognitive deficits using a single therapy (Campos et al, 2016).

In conclusion, early-onset and late-onset AD cannot be considered clinically and/or neuropsychologically different (Vilalta-Franch et al, 2007; Wattmo et al, 2017).

1.2.2. Cholinergic Hypothesis for AD

The cholinergic system includes neurons located in the basal forebrain and their long axons, which reach the cerebral cortex and the hippocampus, modulating cognitive skills. The "cholinergic hypothesis" of AD postulates that functional abnormalities are associated with defective cholinergic neurotransmission. This hypothesis was developed due to the deficiencies in acetylcholine metabolism that were observed in postmortem AD brains (Bohnen et al, 2005).

The alteration of several proteins in the cholinergic system has been reported, such as a decreased activity of choline acetyltransferase (CAT), which significantly correlates with Aβ plaque deposition and cognitive impairment associated mainly with attention and working memory (Bohnen et al, 2005; Dumas et al, 2011; Raskin et al, 2015). Moreover, it has been shown that some macromolecules found in the synapses can interact with Aβ to form protein complexes, which will alter the normal synaptic function. In this regard, Aβ-acetylcholinesterase (AChE) complexes have been demonstrated to be more neurotoxic than Aβ alone, suggesting that AChE may play a key role in the neurodegenerative changes observed in the AD brain (Inestrosa et al, 1996; Muñoz and Inestrosa, 1999). Researchers found a loss of cholinergic neurons in the basal forebrain, correlating with the impairment of cognitive functions and the behavioral disturbances observed in AD patients (Dumas et al, 2011). In fact, these progressive loss of

cholinergic neurons leads to the onset of severe behavioral, motor and cognitive impairments (Craig et al, 2011; Orta-Salazar et al, 2014).

1.2.3. The amyloid precursor protein

APP is processed via two main pathways: the amyloidogenic pathway, which yields the production of Aβ peptides, and the non-amyloidogenic pathway. The non-amyloidogenic pathway is initiated by α-secretase releasing a soluble extracellular fragment termed AβPPα and the intramembranous carboxyl-terminal fragment α (CTFα). The non-amyloidogenic pathway starts with the Beta-site APP Cleaving Enzyme 1 (BACE1) producing an extracellular AβPPβ fragment and an intramembranous CTFβ. Later, γ-secretase cleaves CTFs within its transmembrane domain, producing either a 3 kDa product (p3) from CTFα in the non-amyloidogenic pathway or Aβ from CTFβ in the amyloidogenic pathway. Aβ has different lengths (38, 40, 42 or 43 amino acid residues), with Aβ40 being the most abundant species. They form Aβo and fibrils, which are neurotoxic (Haass, et al, 1994; Farber et al, 1995; Cai et al, 2001; Timmers et al, 2017) (**Fig. 6**).

1.2.4. Apoliprotein ε4

Apolipoprotein E (APOE) acts as a ligand for lipoprotein particles in receptor-mediated endocytosis, and it is important in cholesterol transport in and out of the central nervous system (CNS). People with two copies of the APOE ε4 allele have a high risk of acquiring AD (sporadic or familial) with a significantly lower age of onset compared to patients with sAD. In fact, APOE ε4 is the most important genetic risk factor associated with AD (Liao et al, 2017). APOE ε4 has multiple cellular origins and multiple structural and biophysical properties that might contribute to the pathology of AD through several different mechanisms (Huang et al, 2004). The various mechanisms proposed for APOE ε4 in AD are reviewed below.

APOE regulates Aβ clearance, and endocytic receptors and related molecules for APOE metabolism have been extensively studied. APOE mediates cholesterol efflux to lipid-free apolipoprotein A-I (APOA-I), and it is an essential regulator of high-density lipoproteins (HDL). Targeting transcription factors such as peroxisome proliferator-activated receptor (PPAR), nuclear receptors, liver X receptors (LXRs) and retinoid X receptor (RXR) by using specific

agonists increases the levels of ATP-binding cassette transporter (ABCA1). This transporter is a major regulator of cellular cholesterol and phospholipid homeostasis and decreased Aβ accumulation in APP mouse models (Nam et al, 2016; Liao et al, 2017).

ABCA7, a homologue of the major lipid transporter ABCA1, is highly expressed in neurons and microglia in the brain, constituting one of the most important susceptibility genes in sAD because loss of its function increases the disease risk. It has also been reported that ABCA7 deficit exacerbated brain Aβ deposition in APP/PS1 mice (Sakae et al, 2016).

Low-density lipoprotein receptor (LDLR) and lipoprotein receptor-related protein 1 (LRP1) are the major cell surface receptors for APOE, and some polymorphisms of LRP1 have also been proposed to increase the risk of AD due to decreased Aβ clearance (Koldamova et al, 2014). In fact, APOE ε4 could prevent Aβ clearance, showing less effectiveness in promoting receptor-mediated Aβ clearance than APOE ε3, which would favor Aβ plaque formation and eventual development of AD (Huynh et al, 2017).

APOE ε4 could increase the risk of AD at least in part by binding to an intermediate aggregated form of Aβ and initiating and accelerating aggregation of Aβ (Stratman et al, 2005). On the other hand, several studies confirm that APP proteolysis pathways are modulated at least in part by APOE ε4 because it is able to decrease secreted sAPPα and because all apoE isoforms inhibit Aβ aggregation *in vitro* (Baum et al, 2000). Although APOE ε3 and APOE ε4 may be associated with APP in the nanomolar range, only ApoE ε4 significantly reduces the ratio of sAPPα (Tai et al, 2014), and it also reduces the expression of Silent Information Regulator 2 Homologue 1 (Sirt1), a protein implicated in neuroprotection (Theendakara et al, 2013).

APOE ε4 has also been implicated in Tau phosphorylation and APP phosphorylation and in the induction of apoptosis (Huang et al, 2010; Lattanzio et al, 2014). APOE ε4 may act as a transcription factor, binding DNA and modulating the transcription of several genes involved in energy metabolism, inflammation, axon guidance, neuronal survival and cell death, of which approximately half are not regulated by APOE ε3 (Theendakara et al, 2016).

1.2.5. Amyloid cascade theory for Alzheimer's disease

For almost 25 years, the "amyloid cascade hypothesis" has been studied to understand the etiology and progression of AD. This hypothesis suggests that AD is caused by an imbalance

between the production and clearance of Aβ, leading to Aβ accumulation. The increased levels of Aβ aggregation species, Aβo and fibrils, will lead to a loss of spine density and dendritic complexity and the development of the pathological and clinical events observed in AD (Hardy and Higgins, 1992; Hardy, 2006). This hypothesis is supported by the identification of APP mutations in families with autosomal dominant amyloid angiopathy, dementia and typical hallmarks of AD (Goate et al, 1991). Moreover, PSEN1 and PSEN2, the catalytic components of the γ-secretase complex, are two other genes found to be mutated in autosomal dominant AD (Sherrington et al, 1995).

Many researchers have criticized the amyloid cascade hypothesis of Alzheimer's disease for its inconsistencies and failures, such as in predicting disease symptoms or guiding the development of productive therapies (Behl and Ziegler, 2017). Furthermore, there are other processes in AD that seem to be amyloid-independent: loss of DNA integrity, mitochondrial failure, failures in cell cycle regulation and diabetes. A single approach to explain a complex pathology, in which a single biomarker/process is able to explain the clinical manifestation, in this case the presence of Aβ peptide alone, seems inadequate for AD. This is similar to those of other pathological conditions in which the only cause is old age. It is necessary to work in favor of more comprehensive and multidimensional approaches, promoting systems-level biological analysis of diverse proteins, which would provide a more accurate explication of this pathology (Herrup, 2015; Tse and Herrup, 2017; Behl and Ziegler, 2017; Canevelli et al, 2017).

1.2.6. Revisiting the mitochondrial cascade hypothesis of AD

This hypothesis was proposed in 2004 to provide a more accurate explanation for the biochemical abnormalities in the continuous correlation between advancing age and AD risk. The "mitochondrial cascade hypothesis" emerged in response to the growing body of evidence of AD-related mitochondrial dysfunction. As it mentioned above, mitochondria have multiple functions, including roles in Ca^{2+} homeostasis, bioenergetics, metabolism, redox signaling, autophagy, and dysfunction and mutations in mitochondrial DNA together with OS, contribute to aging, which is the greatest risk factor for neurodegenerative diseases. In fact, in all neurodegenerative processes, there are mitochondrial dysfunctions as an early event, and many studies have demonstrated that

Aβ can induce mitochondrial abnormalities (Swerdlow and Khan, 2004; Lin and Beal, 2006; Swerdlow et al, 2014).

Studies using transgenic animal models have demonstrated alterations in mitochondrial enzymes in the AD brain. Fluorodeoxyglucose [^{18}F] positron emission tomography (FDG-PET) studies have shown that in AD, there is a decrease in the glucose signal, an indicator of synaptic activity, loss of neurons, and abnormal function of the remaining neurons. Moreover, altered metabolism with insulin resistance and glucose utilization seems to precede the clinical diagnosis and can be interpreted as an early clinical finding of mitochondrial failure in AD (Sorbi et al, 1983; Mosconi et al, 2005; Piaceri et al, 2012).

The main evidence implicating mitochondrial dysfunction in AD is summarized as follows: *i)* The observation of decreased energy metabolism occurs due to alterations in the key enzymes involved in oxidative phosphorylation (oxphos), a metabolic pathway directed to oxidize nutrients to produce ATP. This is related to decrease neuronal expression of nuclear genes encoding subunits of the mitochondrial electron transport chain (Chandrasekaran et al, 1994). *ii)* Ca^{2+} imbalance, due to impaired buffering capacity and modifications of the ER Ca^{2+} channels, leads to neuronal apoptosis triggered by the calmodulin-dependent kinases and calpains (Kruman et al, 1998; Nixon, 2003). *iii)* Abnormal mitochondrial dynamics have revealed significantly reduced mitochondrial length. In biopsied AD brains, there is likely enhanced fission with an overexpression of dynamin-like protein 1 protein (DRP1) and downregulation of the optic atrophy protein 1 (OPA1) (Knott et a, 2008). *iv)* Mitochondrial biogenesis is regulated by the Sirt1–protein peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) axis, together with Nuclear Respiratory factor erythroid-derived-2 (Nrf2). In hippocampal tissues from AD patients and APP mouse M17 cells, the levels of PGC-1α, Nrf1 and Nrf2 were significantly decreased in comparison with healthy patients and wild-type mice. In this context, PGC-1α overexpression has been shown to be neuroprotective both *in vitro* and *in vivo* in several models for neurodegenerative diseases (Moi et al, 1994; Gu et al, 2012; Jones et al, 2012).

In contrast to these findings, a recent study showed that continuous PGC-1α overexpression was cytotoxic to dopaminergic neurons *in vivo* (Ciron et al, 2012). *v)* Finally, the products of oxidation, such as 4-hydroxynonenal (4-HNE) due to lipid peroxidation, may facilitate the self-assembly of *tau* protein into fibrillary polymers, paired helical filaments

(PHFs), very similar to those found in the brains of AD patients. These results strongly suggest that OS, anomalous mitochondrial dynamics and dyshomeostasis of Ca^{2+}, either by itself or as part of a two-or-more-hit process, can cause neuronal dysfunction and AD (Ito et al, 1994; Garcia-Escudero et al, 2013) **(FIG 7).**

1.2.7. Oxidative stress and Aβ production

OS regulates Aβ production because some transcription factors, such as heat shock factor-1 (HSF-1) and NF-κB, are able to bind to the promoter region of the APP gene and increase its APP expression. BACE1 is also modulated by various redox-sensitive transcription factors, such as specificity protein 1 (SP1), hypoxia-inducible factor 1α (HIF-1α) and NF-κB. Moreover, OS decreases α-secretase activity and promotes the activation of PS1 and PEN-2 from the γ-secretase complex (Chen et al, 2013; Kaur et al, 2015).

1.2.8. Role of Wnt signaling in AD

Wnt proteins are a family of cysteine-rich glycosylated secreted factors that were named due to the fusion of the Drosophila protein "wingless" and the transcriptional activation of the int-1 gene by viral insertion mutation, which is thought to be a key step in mammary tumor induction by the mouse mammary tumor virus (MMTV) (Hardiman et al, 1996). Currently, 19 of the 24 Wnt genes expressing Wnt proteins have been identified in humans, while 80 *Wnt* genes have been identified from genetic studies in humans, mice, *Drosophila, Xenopus*, and zebrafish (Tortelote et al, 2017). Wnt binds to Frizzled (FZ) transmembrane receptors located on the cell surface, leading to the induction of different downstream signaling pathways (De Ferrari and Inestrosa, 2000; Inestrosa et al, 2002). The first is known as the canonical pathway, which regulates gene transcription through β-catenin and the Tcf-Lef transcription factors (Inestrosa and Arenas, 2010). The other pathway is the non-canonical pathway, which modulates intracellular Ca^{2+} and involves the activation of Ca^{2+}-dependent enzymes, such as Calmodulin kinase II (CAMKII), Protein Kinase C (PKC) and the phosphatase Calcineurin (Kuhl et al, 2000; Kuhl, 2004; Acebron and Niehrs, 2016).

The Wnt signaling pathway is involved in several key cellular processes associated with cellular proliferation, differentiation, adhesion, survival, neurogenesis and apoptosis in different

cell types, including neurons and glial cells (Zhang et al, 1998; Garrido et al, 2002; Rosso and Inestrosa, 2013; Varela-Nallar and Inestrosa, 2013).

Numerous studies have shown that Wnt signaling components are altered in AD (Zhang et al, 1998; De Ferrari and Inestrosa, 2000; De Ferrari et al, 2003). These results suggest a strong relationship between impaired Wnt signaling activity and the neuronal loss observed in AD. Direct Aβ binding to the extracellular FZ receptor, CRD-FZ5, at or near the Wnt-binding site can inhibit this signaling pathway, establishing a direct relationship between Aβ, Wnt and AD (Magdesian et al, 2008; Wang et al, 2016; Arrázola et al, 2017). It was also shown that β-catenin levels are reduced in AD patients carrying inherited PSEN1 mutations (Zhang et al, 1998). PSEN1 can inhibit Wnt signaling and stimulate increased turnover of β-catenin. Disheveled (Dvl), a downstream transducer of Wnt signaling, controls α-secretase cleavage of APP through an interaction with PKC, leading to the translocation of APP from the cytosol to the membrane and may inhibit Aβ production. These effects were accompanied by an increase in the level of the inactive isoform of GSK-3 because activation of PKC provides beneficial effects on the main AD hallmarks, tau and Aβ (Garrido et al, 2002, Alonso et al, 2013). Interestingly, the secreted Wnt antagonist Dickkopf 1(Dkk1) was found to be elevated in postmortem AD brains as well as in brains of transgenic mouse models of AD (Caricasole et al, 2004).

A variant of the low-density lipoprotein-related receptor protein (LRP6) that confers low levels of Wnt signaling activity has been associated with sAD (De Ferrari et al, 2007). Epidemiological data show an increased risk for AD in populations where Apo ε4 is present. Interestingly, ApoE ε4 inhibits canonical Wnt signaling in PC12 cells upon stimulation with the ligand Wnt7a (Caruso et al, 2006).

Synaptic failure is an early event in AD due to the presence of soluble Aβo (Cerpa et al, 2010). Several studies have highlighted the neuroprotective potential of Wnt signaling activation against the toxicity of the Aβ peptide. The ligand Wnt3a was indeed able to overcome toxic effects induced by Aβ in hippocampal neurons (Alvarez et al, 2004). This mechanism is mediated by the receptor FZ1, since this effect is modulated by the expression levels of FZ1 in both PC12 cells and hippocampal neurons (Chacon et al, 2008). Overexpression of FZ1 significantly increased cell survival induced by Wnt3a ligand and decreased caspase-3 activation, while

knocking down the expression of these receptors significantly decreased the levels of β-catenin and reduced the neuroprotective effect elicited by this Wnt ligand (Chacon et al, 2008).

The non-canonical Wnt/Ca^{2+} pathway is activated principally by the ligand Wnt5a (Sowers et al, 2013). Moreover, Wnt5a also promotes PSD-95 protein clustering and the formation of dendritic spines and synaptic boutons in cultured hippocampal neurons (Farias et al, 2009). Recently, we demonstrated that Wnt5a induces NO production, probably by nNOS stimulation, and this NO promotes the insertion of the GluN2B subunit of the NMDAR into the neuronal cell surface by increasing NMDAR trafficking (Muñoz et al, 2014).

An additional effect of the NO-induced up-regulation of GluN2B translation is inducing the phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2α) (Ramos-Fernández et al, 2016). Electrophysiological analysis of Schaffer collaterals in CA1 glutamatergic neurons of the hippocampus has identified a decrease in the amplitude of the field excitatory postsynaptic potential (fEPSP) and excitatory postsynaptic current (EPSC) induced by Aβo, and this decrease can be reversed by activating the Wnt canonical pathway through Wnt5a, thus preventing synaptic damage (Cerpa et al, 2010).

All these findings suggest that the activation of the Wnt/Ca^{2+} pathway improves or protects synaptic function, which is altered in the presence of Aβ (Cerpa et al, 2010; Cerpa et al, 2011). These studies provide additional support for the hypothesis that alterations in the Wnt pathway are involved in the development and progression of AD. Additionally, the activation of several signaling pathways that interact with the Wnt pathway, including the pathways associated with the nicotinic and muscarinic ACh receptors, PPARα and -γ, antioxidants, and anti-inflammatory molecules, support the neuroprotective potential of the Wnt cascade in AD (Farias et al, 2004; Inestrosa and Toledo, 2008; Varela-Nallar et al, 2012; Godoy et al, 2014).

1.2.9. Mitochondrial metabolism involved in neurodegenerative disease

Studies have been focused on understanding the role of mitochondrial dysfunction in neurodegenerative diseases, where a hypothetical sequence of the pathogenic stages would link aging with sporadic AD (Müller et al, 2010). Mitochondria are organelles with high metabolic

rates that combine nutrient metabolism and signaling pathways to regulate health span and longevity by maintaining energy production, Ca^{2+} homeostasis and reducing apoptosis. In neurons, mitochondria are mainly located in the synaptic region, with real importance in dendritic morphogenesis and spine plasticity, and synaptic impairment of mitochondrial function is associated with synaptic deficits, including reduced synapse formation and impaired neuritogenesis (Li et al, 2004).

The brain undergoes structural atrophy and lower energy metabolism in MCI and AD. In fact, the decline in baseline glucose metabolism is a useful tool for monitoring changes in cognition and functionality in AD (Mosconi, et al, 2005; Croteau et al, 2017).

As noted above, the decrease in AD cerebral metabolism is correlated with decreased activity of mitochondrial proteins necessary for energy production, such as pyruvate dehydrogenase complex (PDHC), alpha-ketoglutarate dehydrogenase complex (KGDHC), and isocitrate dehydrogenase (kish, 1997), while other proteins show higher activity, such as succinate dehydrogenase (SD) and malate dehydrogenase (MDH) (Bubber et al, 2005). Several proteins have been identified as directly oxidized and modified in AD, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aconitase, voltage-dependent anion channel (VDAC), ATP synthase-alpha chain and lactate dehydrogenase (LDH). All these changes reveal a reduced number of mitochondria, which will yield defective axonal transport and synaptic degeneration (Bosetti et al, 2002; Sultana and Butterfield, 2009).

This finding was corroborated by morphometric analysis of mitochondrial samples obtained at biopsy from AD patients, demonstrating a significant decrease in mitochondria from neurons in AD brains (Baloyannis, 2006). The normal physiological functions of the mitochondria depend on their structure remaining intact to maintain the electrochemical gradient. Structurally damaged mitochondria, as evidenced by partial or near complete loss of the internal structure and cristae, represent a prominent feature of dystrophic neurons in postmortem AD brains (Ito et el, 1994). These neurons showed increased mitochondrial degradation products, suggesting either greater turnover of mitochondria by autophagy or a reduction in proteolytic turnover, leading to accumulation of mtDNA and mitochondrial proteins (Hirai et al, 2001).

A triple transgenic AD mouse model, a cross of P301L tau transgenic pR5 mice and APPswPS2N141I double-transgenic APP152 mice, showed deregulation of 24 proteins, one-third

of which were mitochondrial proteins mainly related to complexes I and IV of oxphos; specifically, the deregulation of complex I was tau dependent, whereas deregulation of complex IV was Aβ dependent at both the protein and activity levels (Rhein et al, 2009) (**Fig. 8**).

According to mitochondrial aging theory, mtDNA mutations accumulate over time, and these mutations eventually induce ETC dysfunction, enhanced ROS production, decreased mitochondrial membrane potential (MMP) and decreased ATP levels, most likely due to the alteration of the activity of complex I and IV. Notably, this functional decline may activate a compensatory upregulation of mitochondrial mass, and this exacerbation will lead finally to synaptic failure, apoptosis, and neurodegeneration (Calkins et al, 2011).

Energy and Ca^{2+} fluctuations within neuronal synapses are required for neuronal communication, and to achieve this commitment, synapses are enriched in mitochondria for site-directed energy provision and Ca^{2+} homeostasis. In fact, calcium Ca^{2+} mishandling has been reported in peripheral cells isolated from AD patients, with the ER developing Ca^{2+} overloads due to reduced calcium uptake (Ito, et al, 1994).

Studies show that Ca^{2+} levels are elevated in dystrophic neurites in the region of Aβ deposits in transgenic mice, altering neuronal excitability (Kuchibhotla et al, 2008). Other studies showed that Aβ increases the level of cytoplasmic Ca^{2+} due to an influx of extracellular Ca^{2+} across the cell membrane, thereby rendering neurons more susceptible to glutamate-induced neurotoxicity (Mattson et al, 1992). Aβ also impairs membrane ATPase activity due to lipid peroxidation with compromise neuronal membrane integrity, which will lead to loss of ion and Ca^{2+} homeostasis (Butterfield et al, 1994). Finally, Aβ species can bind to the plasma membrane to form artificial membrane pores, and they can form small annular structures disrupting lipid membranes, suggesting that species may cause weakening or thinning of the plasma membrane (Kayed et al, 2004).

Interestingly, an increase in the autophagy mechanism would be the result of inositol depletion that triggers enhanced clearance of damaged mitochondria (mitophagy) and accelerated mechanisms of mitochondrial biogenesis, inducing mitochondrial dynamics to supply and replace the absent or damaged mitochondria (Toker and Agam, 2014; Shah et al, 2015; Gomez-Suaga et al. 2017).

1.2.10. Antioxidants, mitochondria and therapeutics in neurodegenerative diseases

Based on the information presented above, efficient reduction of OS by the activation of multiple antioxidative defense systems or by using antioxidant molecules should be a good strategy in AD. Recent development of antioxidants that selectively concentrate in mitochondria provides a tool and an opportunity to study the impact of mitochondria-generated ROS, especially in synaptic degeneration, and their relationship with neurodegenerative disease (Duchen, 1999). Ideally, mitochondria-targeted antioxidants should be small molecules, pharmaceutically stable, with oral bioavailability and selectively taken up and concentrated by the mitochondria of the neurons or in the organ where they will control oxidative damage (Smith and Murphy, 2010; Korshunova et al, 2017). These molecules should be focused on antioxidant activity, in order to restore the normal physiological function of mitochondria, improving their biogenesis, modulating mitochondrial dynamics or promoting mitochondrial networking yielding to repair and regenerate neurons, to promote axonal transport, and to enhance synapse formation and synaptic activities in AD neurons (Manczak et al, 2010; McManus et al, 2011). In fact, antioxidants such as vitamin E, vitamin D, curcumin, *Ginkgo biloba* compounds and melatonin have been shown to reduce soluble Aβ levels and improve mitochondrial function and cognitive behavior in animals (Reddy et al, 2012; Grimm et al, 2016).

1.2.10.1. Keap1 and Nrf2

Another strategy to preserve redox balance and adequate mitochondrial function is the induction of endogenous antioxidant mechanisms, among which the most sensitive is Kelch-like ECH-associated protein1 (Keap1). It is a key sensor for the adaptive stress response system regulated through the transcription factor Nrf2, which forms a complex with Keap1, this protein act as a repressor protein in the cytoplasm and plays an essential role in Nrf2 degradation by the ubiquitin–proteasome pathway (Harder et al, 2015). Keap1 acts as a major sensor for OS. A Cys residue is alkylated in Keap1, and when oxidized, it changes the secondary structure of Keap1, which allows the two proteins to dissociate. Nrf2 translocates to the nucleus to start the transcription of several genes directly via an antioxidant response element (ARE) to effectively

control OS with strong neuroprotective and anti-inflammatory activity (Hur and Gray, 2011; Deshmukh et al, 2017). The Nrf2-regulated genes are γ-glutamyl cysteine ligase (γ-GCL), NAD(P)H quinone oxidoreductase-1 (NQO-1), glutathione S-transferase (GST), heme oxygenase-1 (HO-1), uridine diphosphate (UDP) glucuronosyl transferase (UGT1A1), SOD1, catalase and plasma glutathione peroxidase (GPX-3) (Park et al, 2004; Hur and Gray, 2011).

1.2.10.2. PPARγ

Increasing interest has recently been focused on PPARγ, this type of receptor was initially identified as a key regulator of transcription in adipogenesis, but it was also shown to play additional roles in other biologically relevant processes such as infection and inflammation (Zhang et al, 2015). The activation of PPARγ attenuates the inflammatory response and improves cognitive dysfunction associated with many neurodegenerative disorders. Decreased PPAR-γ expression in aged individuals has also been reported; that would be involved in increased neuroinflammation and the consequent reduction of inhibitory redox-regulated transcription factors (Zhang et al, 2017; Zolezzi et al, 2017).

PPARγ is a negative regulator of OS by transcriptional repression of many well-characterized proinflammatory transcription factors and enzymes such as NF-κB, signal transducer and activator of transcription-6 (STAT-6), activator protein-1 (AP-1), cyclooxygenase-2 (COX-2) and inducible NOS (iNOS) (Valenzuela et al, 2017). It triggers the expression of several antioxidant genes, such as HO-1, catalase, GPX-3 and manganese superoxide dismutase (MnSOD). Several studies have suggested the existence of reciprocal regulation between the Nrf2 and PPARγ pathways to reinforce each other's expression, and the two pathways seem to be connected by a positive feedback loop that maintains the simultaneous expression of both transcription factors and their target antioxidant genes and protects against oxidative injury (Cho et al, 2005; Reddy and Standiford, 2010). The Nrf2 shows activity on the PPARγ gene, and collaborative activity of both Nrf2 and PPARγ transcription factors on a single target gene also seems to be plausible because the GST promoter was found to possess both ARE and PPAR response element (PPRE) sequences to allow simultaneous stimulation of its transcription (Cho et al, 2010; Zhao et al, 2015).

PPARγ has thus emerged as a new target for anti-inflammatory and antioxidative pharmacotherapy in various diseases that show OS and inflammation (Chung et al, 2009; Lee, 2017; Zolezzi et al, 2017). The strategies aimed to activate PPARγ and Nrf2 should be protective regarding neurodegeneration (Moi et al, 1994; Zolezzi et al, 2017). Pioglitazone, a well-known PPAR-γ agonist that is used in the treatment of type 2 diabetes mellitus (T2DM), has vascular protective effects and anti-aging properties. It produces an increase in glucose catabolism, the upregulation of uncoupling protein 2 (UCP2), a transporter protein present in the mitochondrial inner membrane, and the inhibition of NADPH oxidases, thereby lowering the levels of $O^{\cdot -}$ (Bagi et al, 2004; Wang et al, 2014).

1.2.10.3. Flavonoids

Flavonoids, a group of plant-derived phenolic compounds, have also been reported to have protective effects on neuronal cells (Shadfar et al, 2015). Consumption in the diet benefits cardiovascular health in humans and delays the onset of chronic aging-related diseases (Paterniti et al, 2014). Since many flavonoids are known to act as free radical scavengers, putative health benefits were partly attributed to their direct antioxidant capacity.

However, it has become apparent that flavonoids modulate signaling processes in cultured cells and possibly also *in vivo* because they possess the ability to augment intracellular reduced glutathione (GSH) and the ability to prevent lipid peroxidation in a cell model of AD (Jimenez-Aliaga et al., 2011; Park, 2010; Bui and Nguyen, 2017).

Quercetin, one of the most abundant dietary flavonoids, activates Nrf2, PPARγ and forkhead box O (Foxos) (Arredondo et al, 2010; Pallauf et al, 2017). Quercetin has protective functions by inducing c-Jun N-terminal kinase (JNK) in hepatotoxicity, inhibiting the binding of Keap1 to Nrf2 and thus leading to increased expression of antioxidative genes downstream of Nrf2 (Ji et al, 2015). Quercetin and rutin, another flavonoid, inhibited the formation of Aβ fibrils, disaggregated Aβ fibrils, increased proteasome activity and reduced the activity of caspase-3 (Martín-Aragón et al, 2016). Extensively studied compounds with cancer-chemopreventive activity are widely recognized as effective antioxidants; however, quercetin has

a low antioxidant activity but induces the long-term "hormetic" Nrf2, a dose-response phenomenon characterized by stimulation at low doses and inhibition at high doses (Zhang et al, 2008; Stępkowski et al, 2011). Therefore, the dose is important, and the induction of apoptosis by polyphenolic compounds such as quercetin is proposed to inhibit NF-κB and to activate the apoptosis signal-regulating kinase (ASK1)/p38 pathway. This pathway, together with AMP-activated protein kinase (AMPK), seems to be a critical controller of quercetin-regulated ASK1/p38 activation, suggesting that quercetin-exerted apoptotic effects involve the ROS/AMPKα1/ASK1/p38 signaling pathway (Lee et al, 2010; Zhang et al, 2010).

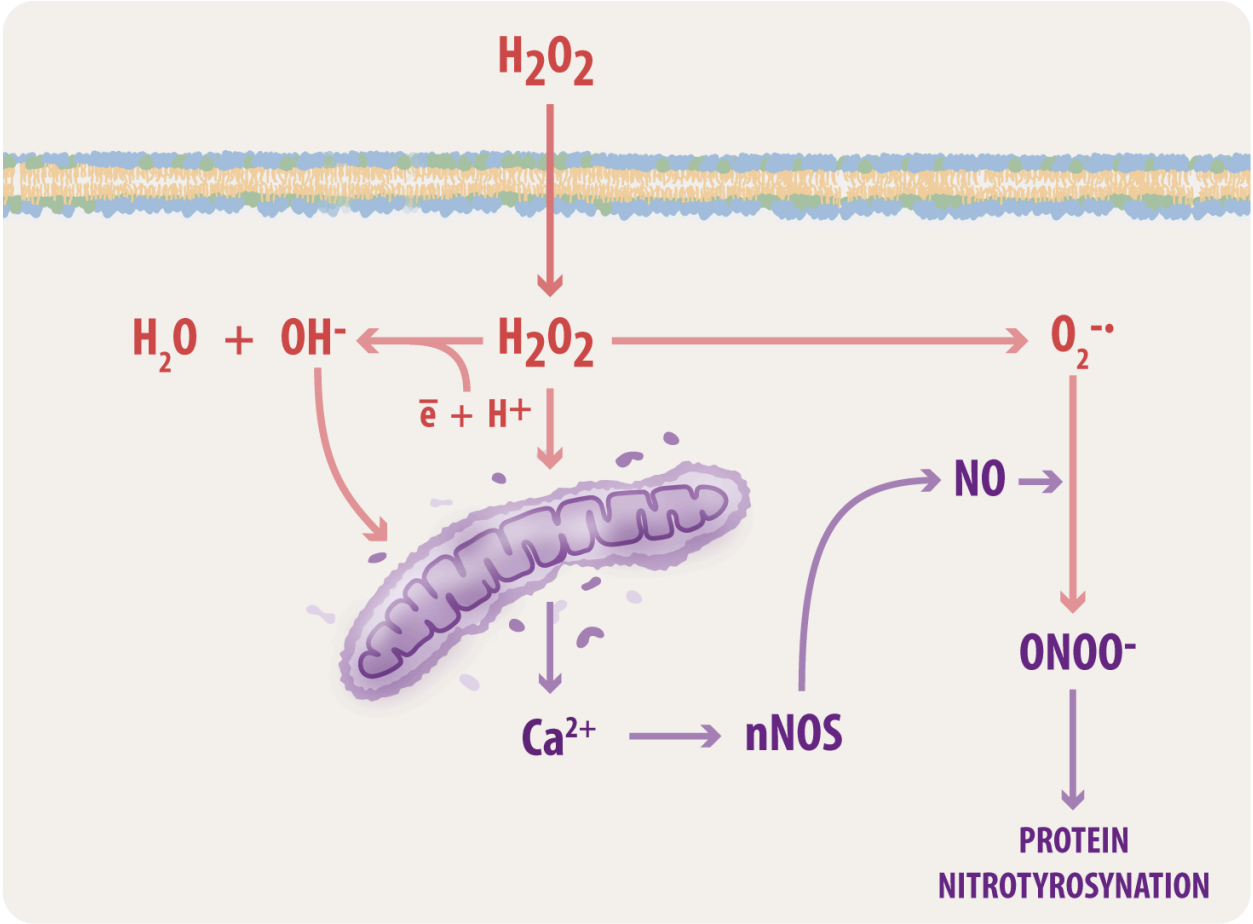

Fig. 1. ROS production. One source of ROS under normal conditions in humans is the leakage of activated oxygen from mitochondria during oxidative phosphorylation. This occurs when unpaired electrons escape from the ETC and react with molecular oxygen, generating various types of ROS. These ROS are superoxide (O^{-}_{2}) and hydroxyl ($OH^{\cdot}$), as well as the non-radical species hydrogen peroxide (H_2O_2). Nitric oxide (NO) can react with O^{-}_{2} to form highly reactive peroxynitrite (ONOO-).

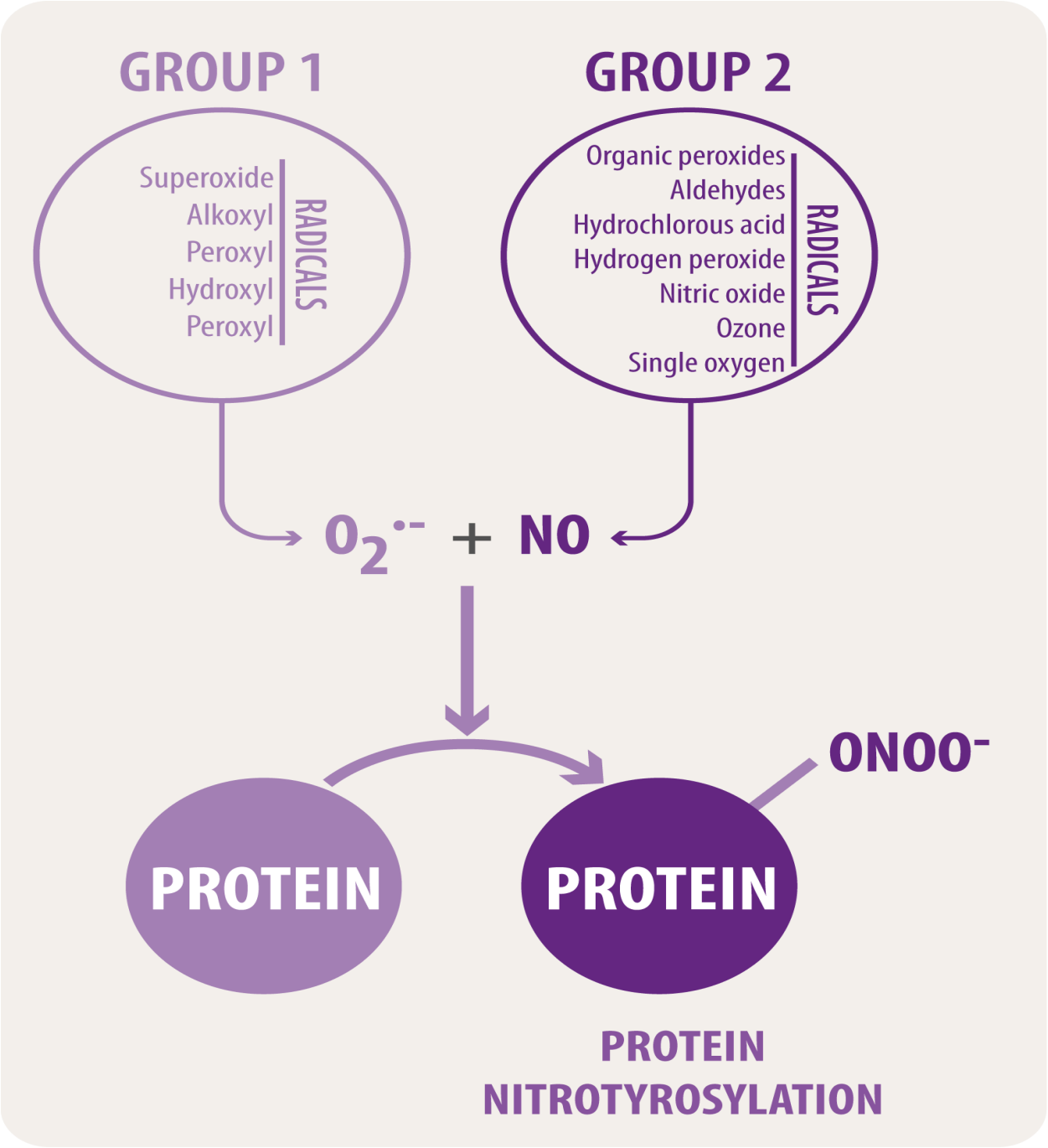

Fig. 2. Protein nitration. ROS can react with NO, the product of neuronal nitric oxide synthase (nNOS), generating reactive nitrogen species (RNS), such as peroxynitrite (ONOO−). It reacts with the tyrosines of proteins in a process termed nitrotyrosylation.

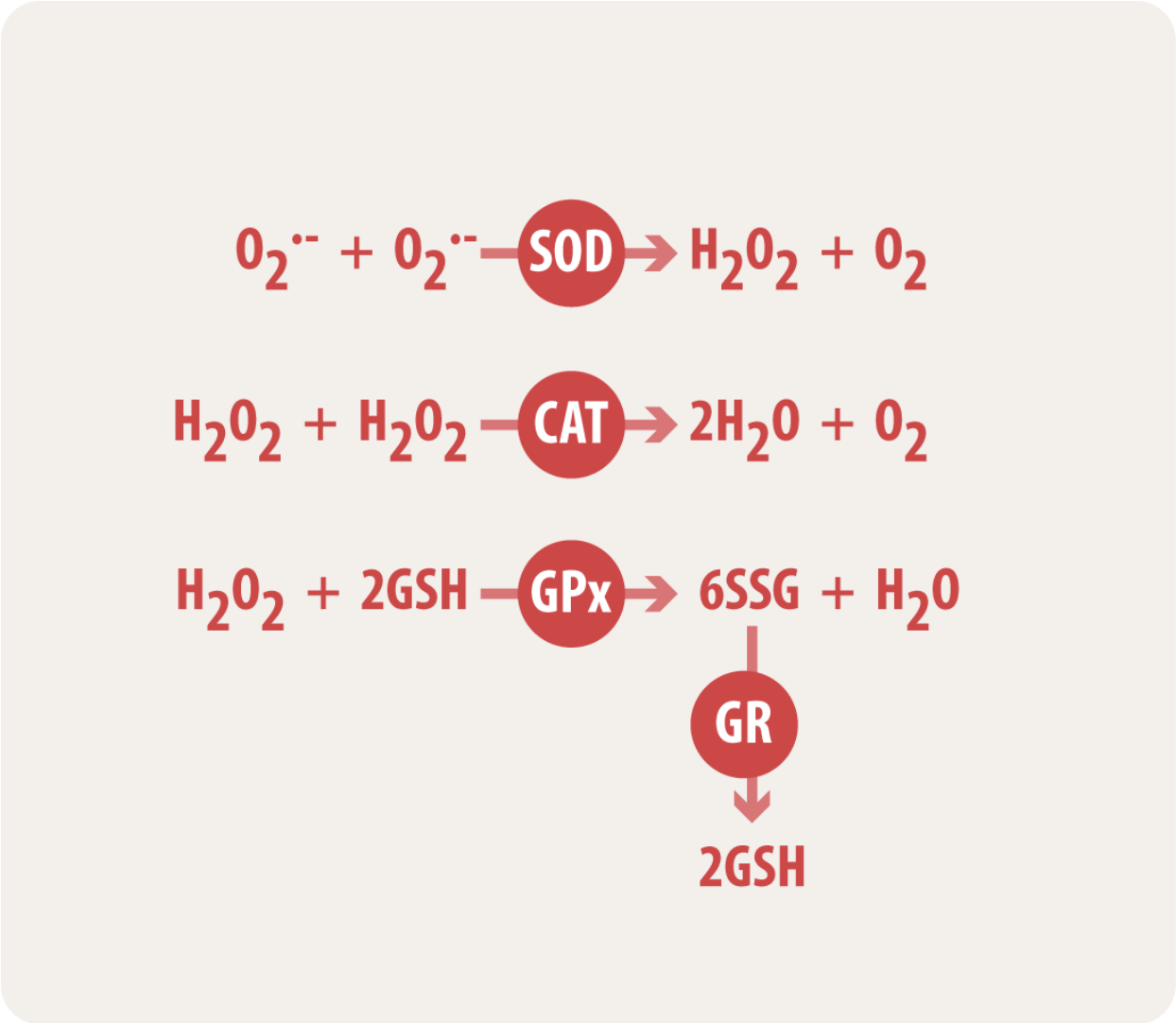

Fig. 3. ROS generation and regulation. Oxidative stress is caused by a general progressive imbalance between the generation of intracellular ROS produced in mitochondria and pathways involved in antioxidant defense, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

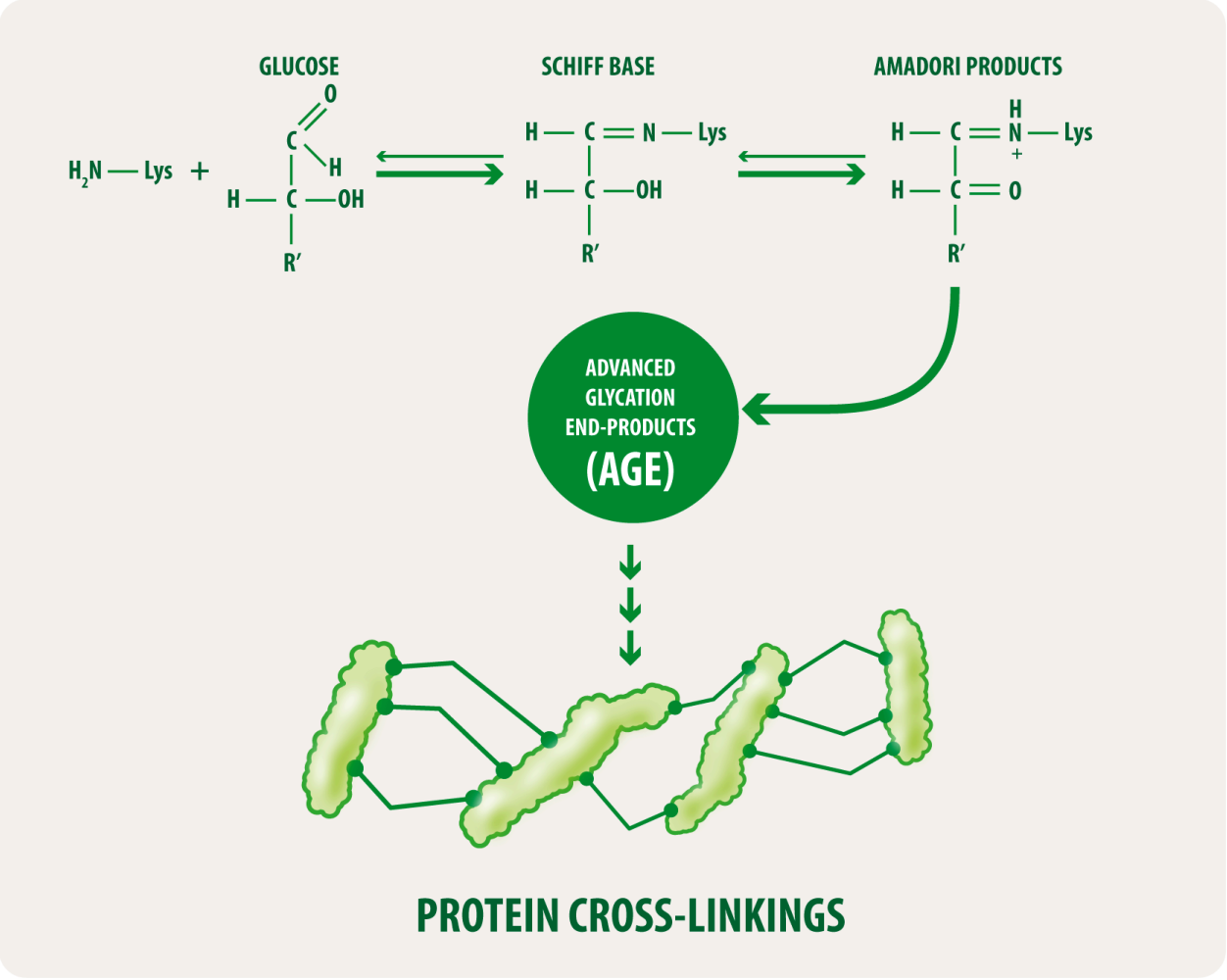

Fig. 4. Non-enzymatic glycation of proteins. Proteins can undergo modifications by binding sugars and producing glycation and Amadori products with the consequent inactivation of the proteins.

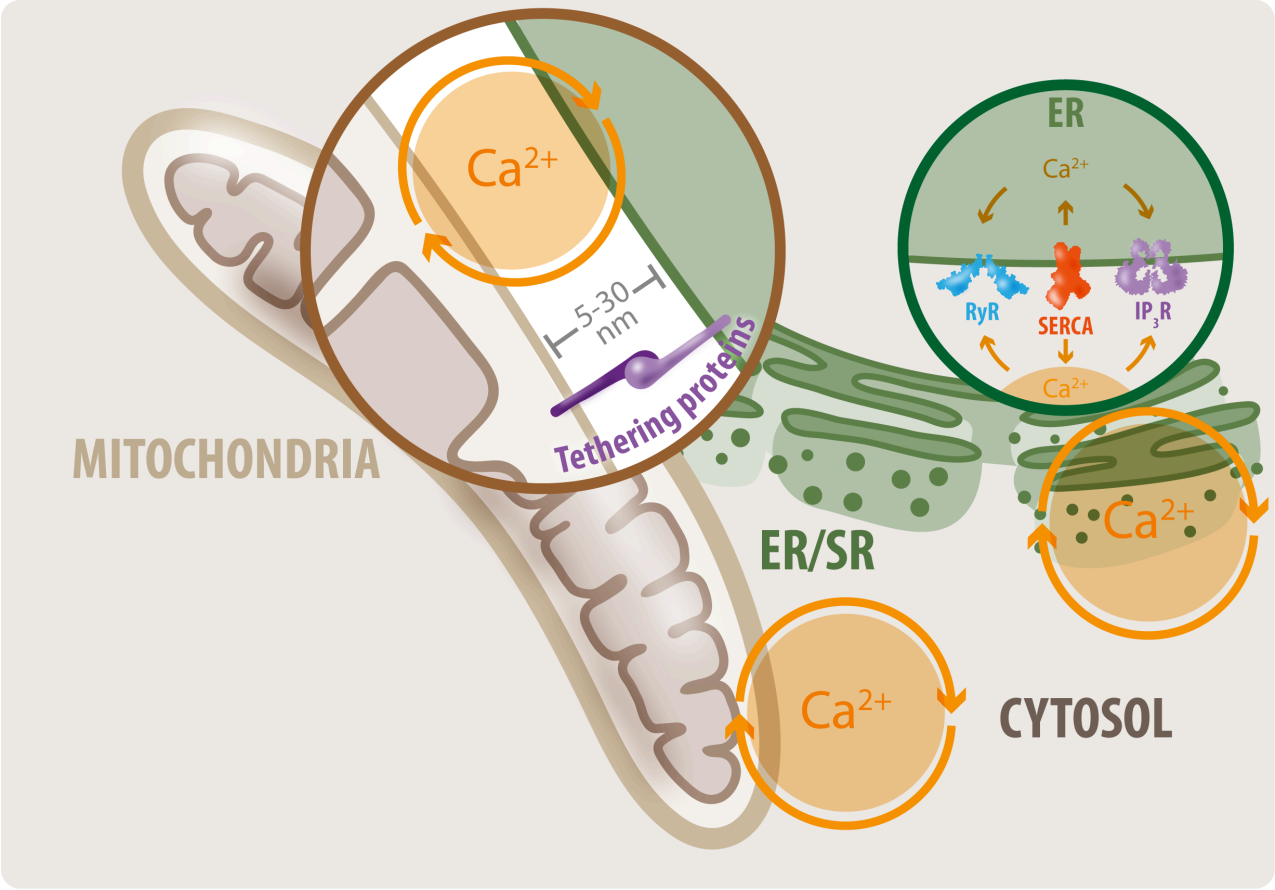

Fig. 5. The mitochondria anchored to the ER allow the exchange of calcium. Cytosolic Ca^{2+} regulates IP_3-induced Ca^{2+} release (IICR). IP_3 receptors (IP_3R) and ryanodine receptors (RyR) are located in the endoplasmic reticulum (ER). This activity shows a typical biphasic pattern with a flow from a region with a low Ca^{2+} concentration toward a region with a higher inhibitory concentration.

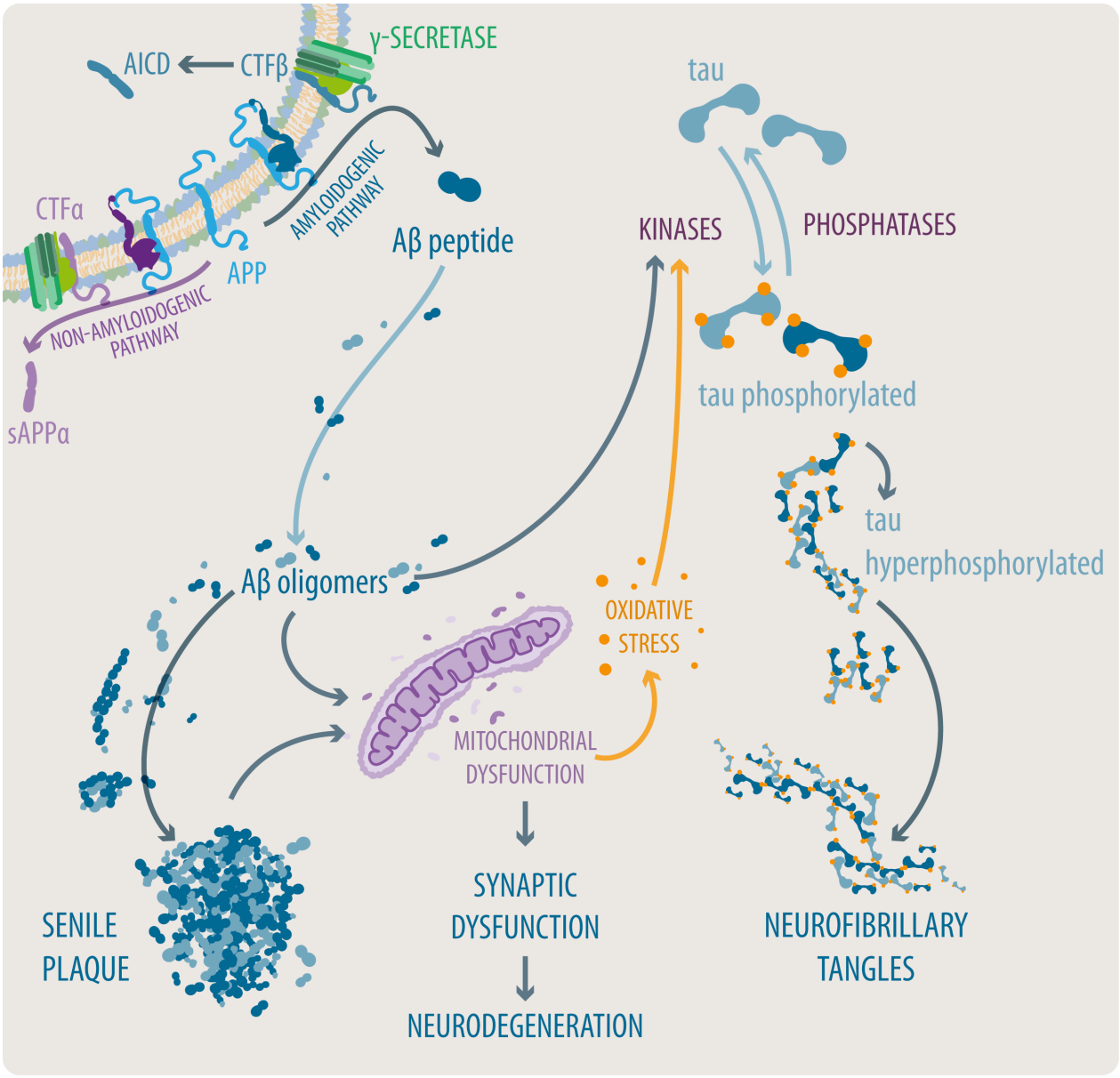

Fig. 6. APP processing, synaptic dysfunction and AD. The neuropathology of AD includes the accumulation of extracellular β-amyloid (Aβ), forming senile plaques and intraneuronal neurofibrillary tangles (NFTs) constituted mainly by hyperphosphorylated tau protein. Aβ leads to mitochondrial dysfunction and the loss of neurons and synapses correlating with the neurodegeneration. AICD, The amyloid precursor protein intracellular domain; CTF, alpha- or beta-secretase-cleaved COOH-terminal fragments of APP.

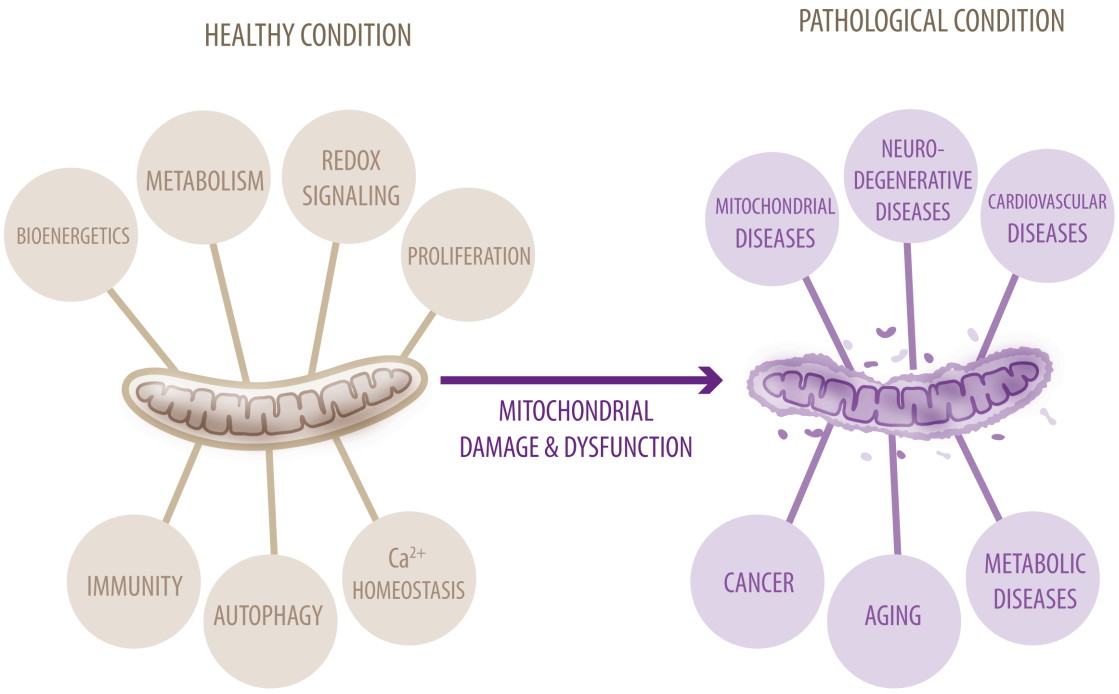

Fig. 7. Mitochondrial cascade hypothesis. Mitochondria have multiple functions in bioenergetics, metabolism, redox signaling, proliferation, immunity, autophagy and Ca^{2+} homeostasis. Dysfunction and mutations in mitochondria, together with oxidative stress, contribute to aging, the most important risk factor for neurodegenerative diseases. These changes strongly suggest that oxidative stress, anomalous mitochondrial dynamics and dyshomeostasis of Ca^{2+}, either by itself or as part of a two-or-more-hit process, can cause neuronal dysfunction and AD.

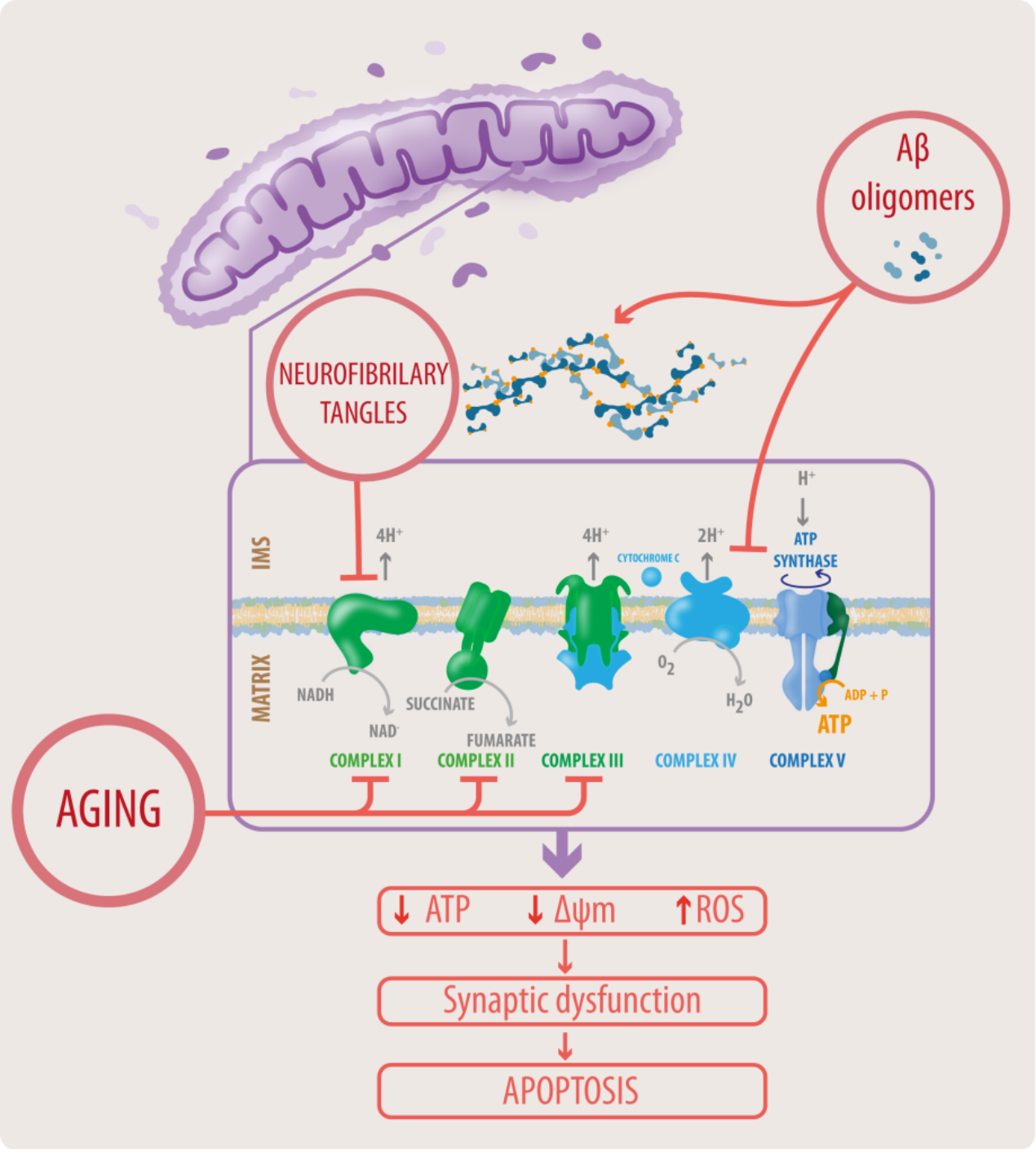

Fig. 8. Mitochondrial aging theory for neurodegenerative disease. Deregulation of mitochondrial proteins mainly related to complexes I and IV of the oxphos system, showing the deregulation of complex I in a tau-dependent manner. Deregulation of complex IV is Aβ dependent at both the protein and activity levels. Mutations accumulate over time, and these mutations eventually induce ETC dysfunction, enhanced ROS production, decreased mitochondrial membrane potential (MMP) and decreased ATP levels. The impairment of these functions exacerbates synaptic failure, apoptosis and neurodegeneration.

III. OBJECTIVES

Aging of the brain is the key step in the development of neurodegenerative diseases such as AD, dramatically affecting the hippocampus and thus impairing memory and learning.

Aging is a process characterized by increased oxidative stress. Free radicals induce damage to biomolecules that will impair the proper function of subcellular organelles and therefore of the cells. Neurons are particularly affected by oxidative stress, and those effects alter cognitive functions such as memory that depend on proper hippocampal function.

Mitochondria are subcellular organelles that produce ATP, but they also perform other cellular regulatory functions, such as calcium homeostasis, lipid metabolism and cell metabolism. Therefore, the maintenance of a healthy population of mitochondria is needed for the proper function of neurons. Mitochondrial dynamics is a process consisting of the physiological fusion and fission of the pool of mitochondria depending on the different cell requirements, but it can be affected by aging and AD.

Antioxidant activity has been proposed to retard the effects of systemic aging and to protect neurons against Aβ toxicity. Antioxidants are found in a wide range of foods, and they can cross the blood–brain barrier. The most interesting antioxidants would work at low concentrations, below micromolar (μM) range, which is rare for classic antioxidants. Therefore, even with proper antioxidant activity, these molecules should target specific systems to detoxify cells, such as antioxidant intracellular enzymes or other neuroprotective intracellular signaling pathways. Quercetin is a natural flavonoid present in garlic, onions, blueberries and green tea and is an excellent candidate that meets the requirements for being a therapeutic antioxidant.

On the other hand, AD has a close relationship with insulin resistance and neuroinflammatory processes. The transcription factor PPARγ is a target considered highly relevant in AD since PPARγ decreases hyperglycemia, but it is also directed toward protection against metabolic syndrome, neuroinflammation and oxidative stress. Synthetic drugs that are selective peroxisome proliferator-activated receptor modulator agonists (SPPARMs) are being tested clinically (Motani et al, 2009; Higgins et al, 2010). Therefore, we have studied the role of PPARγ agonists in the protection against Aβ in hippocampal neurons.

The main aim of the present doctoral thesis is to study mitochondrial dynamics in aging and AD. From this general objective, we have defined the following particular objectives:

1. To study the antioxidant and protective activities of a flavonoid, quercetin, in cultured hippocampal neurons and their effect on mitochondria undergoing OS- and Aβ-induced degeneration.

2. To study the effects of SPPARMs on cultured hippocampal neurons challenged with Aβ.

To meet with these objetives, we used INT131and rosiglitazone and analyzed their protective roles in the mitochondria.

IV. MATERIALS AND METHODS

All the experiments carried out throughout this doctoral thesis have been exhaustively described in the articles. Here, I justify the biological material used in the works.

4.1. *In vitro* hippocampal neuron model

The hippocampus is the primary brain region involved in memory formation and learning. Both functions are affected in physiological aging and AD. Therefore, we have performed all the experiments in primary cultures of rat hippocampal neurons at 14 days *in vitro* (14DIV). At that time, neurons are mature and polarized, expressing all the functional receptors required for their proper function. Moreover, in this cell model, we can study the structure and morphology of the dendritic tree and the mitochondrial dynamics along the neurites. The isolation and culture of rat hippocampal neurons is detailed in the articles included in the Results section.

4.2. Amyloid β-peptide oligomers

Because the most toxic forms of Aβ are the Aβo and because they are proposed to induce damage in the synaptic cleft and along the neuronal surface, we have performed all the experiments with Aβo. They have been prepared as indicated in the articles included in the

Results section. In the first article (Godoy et al., 2017), we characterized the Aβo by western blotting and transmission electron microscopy.

4.3. SOD2$^{+/-}$ mice

The homozygous transgenic mouse line B6.129S7-Sod2tm1Leb/J was obtained from the Jackson Laboratory (Stock no. 002973). We used heterozygous mice because these animals show diminished antioxidant status with a 79% decrease in MnSOD levels in the brain. Wild-type and treated animals were housed in the Pontifical Catholic University animal facility. All animal procedures were approved by the Ethical Committee for Animal Research.

V. RESULTS

5.1. Quercetin Exerts Differential Neuroprotective Effects against H_2O_2 and Aβ Aggregates in Hippocampal Neurons: the role of Mitochondria.

In this article, we aimed to study the protective role of quercetin against oxidative stress and Aβ challenge, as described in objective 1.

Godoy JA, Lindsay CB, Quintanilla RA, Carvajal FJ, Cerpa W, Inestrosa NC. Quercetin Exerts Differential Neuroprotective Effects Against H2O2 and Aβ Aggregates in Hippocampal Neurons: the Role of Mitochondria. Mol Neurobiol. 2017 Nov 28;54(9):7116–28. DOI: 10.1007/ s12035-016-0203-x

5.2. INT131 increases dendritic arborization and protects against Aβ toxicity by inducing mitochondrial changes in hippocampal neurons.

In this article, we aimed to study the protective roles of rosiglitazone and INT131 against Aβ challenge, as described in objective 2.

Godoy JA, Zolezzi JM, Inestrosa NC. INT131 increases dendritic arborization and protects against Aβ toxicity by inducing mitochondrial changes in hippocampal neurons. Biochem Biophys Res Commun. 2017 Aug 26;490(3):955–62. DOI: 10.1016/j.bbrc.2017.06.146

5.3. PPARs in the central nervous system: roles in neurodegeneration and neuroinflammation.

In this review, we have summarized the current knowledge of the neuroprotective effects of PPAR in the central nervous system.

Zolezzi JM, Santos MJ, Bastías-Candia S, Pinto C, Godoy JA, Inestrosa NC. PPARs in the central nervous system: roles in neurodegeneration and neuroinflammation. Biol Rev. 2017 Nov;92(4):2046–69. DOI: 10.1111/brv.12320

VI. DISCUSSION

6.1. Mitochondrial dynamic, aging and AD

Aging is the most important feature in the development of different age-related neurodegenerative diseases, including AD. Mitochondria and their metabolic functions change dramatically with age, and mitochondrial dysfunction appears to be a critical factor in the pathogenesis of AD.

Mitochondria have been shown to have reduced membrane potential, increased permeability and calcium dyshomeostasis, these pathological events will affect the regulation of mitochondrial dynamics, a process that controls the total number of mitochondria based on the metabolic needs of the cells. Mitochondrial and bioenergetic dysfunction could therefore be a link between aging and neurodegeneration, since most of the mitochondrial defects in AD patients are not limited to the brain (Swerdlow et al, 2017).

In AD, all evidence suggests that overproduction of APP and Aβ may affect mitochondrial dynamic (fusion/fission), shifted towards increased fission. In fact, both APP and Aβ are found in mitochondrial membranes and with mitochondrial proteins, contributing to mitochondrial abnormalities (Manczak et al, 2011).

Pathological conditions such as AD, mitochondria produce ROS and neurons will suffer noxious challenges, mainly due to damage to proteins, lipids, and nucleic acids. Interestingly, in response to several cellular conditions, such as OS, increased energy demand, exercise training, aging and certain neurodegenerative diseases, energetic demands are supplied by increases in the number of mitochondria. These increases are mediated by the specific cofactor PGC-1α. PGC-1α activates various transcription factors, including nuclear-encoded transcription factors such as NRF-1 and NRF-2, which regulate transcription of nuclear and mitochondrial genes involved in oxphos, ETC complexes I-V, mtDNA transcription/replication, protein import and assembly of ion channels (Kelly and Scarpulla, 2004). Proper modulation of mitochondrial dynamics and metabolic function may prevent the generation of excessive ROS, preserve neuronal survival and promote the energy production necessary to maintain healthy mitochondria and eliminate dysfunctional mitochondria. It is necessary to maintain efficient and functional mitochondrial

mass in response to various stressors, including hypoxia and nutrient starvation, which could be relevant in delaying or managing the degenerative process in aging and AD (Karbowski and Neutzner, 2012). Unfortunately, mitochondrial biogenesis is thought to be impaired in AD, and the number of mitochondria and the levels of the transcription factors Nrf1, Nrf2, and TFAM, along with nuclear levels of PGC-1α, are reduced in hippocampal tissues from AD brain (Qin et al, 2009).

6.2. Activities of quercetin in neurons

Quercetin apparently plays key roles in protecting hippocampal CA1 pyramidal neurons from ischemia/reperfusion injury. In animals, pretreatment with quercetin protects hippocampal CA1 pyramidal neurons from this type of injury by increasing the activities of the enzymes SOD1, SOD2, CAT and GPX (Chen et al, 2017).

The effect of quercetin on ion channels has also been described, stimulating Na^+-K^+-2Cl-cotransporter 1 (NKCC1), which is one of the most important ion transporters regulating the cytosolic chloride concentration and controlling cellular functions such as neurite elongation via polymerization of tubulin by inhibiting GTPase activity (Nakajima et al, 2011; Marunaka, 2017).

Quercetin alleviated behavioral dysfunction in a chronic unpredicted stress (CUS) animal model. These animals show high levels of OS markers, such as plasma thiobarbituric acid reactive substances (TBARS); lowered antioxidants, such as total thiol and catalase levels; and enhanced expression of pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β and COX-2, in the hippocampus and damaged hippocampal neurons. Treatment with quercetin significantly normalized locomotor activity, attenuated depression, reduced anxiety and improved cognitive dysfunction (Mehta et al, 2017a). CUS markedly down-regulated insulin signaling in the CA3 region, and quercetin treatment improved insulin resistance and increased neuronal GLUT4, an insulin-dependent type of glucose transporter. These results suggest that insulin function in the hippocampus region is essential for cognitive functions and that quercetin improves CUS-mediated cognitive dysfunction by modulating hippocampal insulin signaling (Mehta et al, 2017b).

AD is also characterized by OS, loss of physiological mitochondrial dynamics and neuroinflammation, it is in this sense that treatment with quercetin could significantly reduce

oxidative damage, being able to effectively prevent neurological complications induced by oxidative stress as well as inflammation. As we have obtained evidence that quercetin prevents the effect of oxidative stress on mitochondrial dynamics, restoring normal mitochondrial dynamics and partially rescuing the noxious effect of Aβ oligomers, we consider that this compound holds promise as a new therapeutic approach to treating AD. The partial effect of Aβ oligomers suggests that other mechanisms triggered by Aβ are working concomitantly.

6.3. Role of PPARγ in neurodegeneration

PPARγ, which belongs to the family of ligand-activated nuclear receptors, controls and regulates the expression of a large number of genes involved in the intermediary metabolism of glucose and lipid, adipogenesis, insulin sensitivity, immune response, cell growth, and differentiation. The specific ligands for this type of receptors are from the group called thiazolidinediones, are known to control many physiological, pathological and inflammatory pathways (Zolezzi et al, 2017). PPARγ agonists such as rosiglitazone could abrogate AGE-mediated neurotoxic effects on human neural stem cells (hNSCs), and in hNSCs, PPARγ may play a role in diabetes-related neuronal impairment. Rosiglitazone can activate the PGC-1α pathway and regulate oxidative defense genes, such as SOD1, SOD2 and Gpx1. The stimulation of mitochondrial function and anti-OS mechanisms by this agonist significantly normalized the inflammatory responses through TNF-α, IL-1β, NF-κB and inflammatory genes such as iNOS and COX-2 (Chiang et al, 2017).

6.4. Diabetes type 3 (T3DM) and PPARγ agonists

T2DM is a metabolic disorder characterized by hyperglycemia in the context of insulin resistance. T2DM is one of the risk factors for AD pathogenesis by impairment in insulin signaling and glucose metabolism in the peripheral system as well as the CNS. The two diseases share common characteristics, such as inflammation and loss of cognitive function. Increased coexistence of AD and T2DM suggests that insulin resistance, impaired glucose and lipid metabolism lead to Aβ aggregation, Tau hyperphosphorylation, mitochondrial dysfunction, OS,

protein misfolding, memory impairment and inhibition of Aβ transport through the blood brain barriers (BBB) (Pardeshi et al, 2017).

There are *in vivo* studies demonstrating that insulin promotes the production of Aβ and that Aβ competes with insulin for the insulin receptor. Insulin itself can aggregate and form fibrils, and it has been demonstrated that insulin interacts with soluble Aβ, decreasing the kinetics of aggregation of one with respect to the other. In fact, Aβo was less cytotoxic when formed in the presence of insulin than Aβo formed without insulin. Moreover, mature Aβ fibrils induced fibrillation of soluble insulin, but insulin aggregates did not promote Aβ fibrillation. The study of this mechanism provided a promising field to investigate alterations in glucose metabolism inside brain or type 3 diabetes mellitus (T3DM), which shared molecular degenerative cascades such as dysfunction in the insulin signaling pathway (de la Monte and Wands, 2008). This molecular interaction between insulin and Aβ and the insulin receptor suggests that direct molecular interactions may contribute to the strong link between T2DM and AD (Luo et al, 2016).

AD and T2DM share common pathological mechanisms, such as enzymatic degradation of Aβ, forkhead box protein O1 (foxo) signaling and insulin signaling (Pardeshi et al, 2017). Aβ levels in animal models are transiently increased by short-term treatment with insulin, generating insulin resistance and decreased total APP. When insulin levels are restored to physiological levels, there is an inhibition of hepatic aPKC and Protein kinase B (PKB), also known as Akt, which are required for insulin-stimulated glucose transport, and it results in the improvement of systemic insulin resistance; consequently, brain insulin signaling is normalized. On the other hand, PGC-1α and foxos are essential for memory, long-term neuronal function and regeneration, and for this reason, the abnormal hyperinsulinemic states may contribute to linking insulin resistance to AD (Farese et al, 2005).

The differences in the activity of several drugs on the metabolic alteration of glucose and lipids could be due to differences caused by their diverse effects on lipid sub-fractions in this sense, rosiglitazone significantly increases HDL levels, total cholesterol and the LDL fraction.

Other agonist as pioglitazone increases HDL cholesterol and decreases triglycerides and fasting plasma free fatty acids but has no effect on total cholesterol or LDL cholesterol. In T2DM, long-term activation of PPARγ by agonist of the thiazolidinediones-type reduces glycemia and hyperinsulinemia, thereby modifying metabolic disturbances, but also attenuates

vascular dysfunction protecting vascular function in diabetes in T2DM mice. Short-term treatment with rosiglitazone augmented NO-mediated flow-dependent dilations of coronary arterioles by reducing vascular superoxide production with favorable oxidant/antioxidant enzyme activities (Grygiel-Górniak, 2014).

Rosiglitazone shows activity against CUS, as previously described, the depression induced in these animals is a risk factor for diabetes and behavioral dysfunctions and treatment with this agonist effectively improved hippocampal insulin signaling, GLUT4 membrane translocation and cognitive performance in depressed mice. Rosiglitazone administration would have beneficial effects on impaired glucose tolerance and neurological disorders associated with depressive-like behavior (Patel et al, 2016).

Regarding Aβ clearance, simultaneous treatment with rosiglitazone and pioglitazone significantly increased Aβ efflux and decreased Aβ influx across the BBB in db/db mice. This process is accompanied by amelioration of amyloidosis with an improvement in hippocampal plasticity in T2DM model mice (Wang et al, 2016). These animals have decreased levels of hippocampal $A\beta_{1-40}$ and $A\beta_{1-42}$ and suppressed neuronal apoptosis, as indicated by decreased caspase-3 activity and an increased Bcl-2/Bax ratio. They also show increased hippocampal plasticity, as characterized by enhanced *in vivo* LTP and better performance on behavioral tests. Furthermore, PPARγ agonists induced the expression of the LRP1 gene by the activation of PPARγ, favoring Aβ clearance (Wang et al, 2017).

Recently, several research groups have demonstrated the effectiveness of the combined use of antioxidants and pharmacological activators of PPAR and the activation of both antioxidant pathways are critical to protect neurons from the etiopathogenic functions of the SO on mitochondrial dynamics, synaptogenesis and neuronal survival, functions that are fundamental in aging and AD. Therefore, efficient reduction of OS through activation of multiple antioxidative defense systems could be a promising strategy to improve a wide range of ROS-induced pathological conditions (Lee, 2017).

In summary, a growing body of evidence strongly suggests that aging and oxidative damage are tightly linked with the pathogenesis of several chronic metabolic disorders such diabetes, atherosclerosis and neurodegenerative disease, especially AD. Therefore, insufficient

cellular protection against OS is another contributing factor in the development of neurodegenerative diseases (Muller et al, 2007; Newsholme et al, 2016).

Our results indicate that quercetin efficiently protected against H_2O_2-induced neuronal toxicity; however, this protection was only partial in rat hippocampal neurons that were treated with Aβ. Treatment with quercetin decreased ROS levels, restored the normal morphology of mitochondria, and prevented mitochondrial dysfunction in neurons that were treated with H_2O_2.

Our work with PPARγ agonists (SPPARM) showed protection of hippocampal neurons against the damage produced by Aβo by suppressing OS and by improving the number of mitochondria and mitochondrial dynamics in neurons. These effects may be mediated by the activation of Nrf2/ARE/HO-1 by quercetin and antioxidant mechanisms activated by PPARγ pathways. Further work is needed to characterize all the mechanisms involved in the neuroprotection demonstrated by quercetin and PPARγ agonists.

VII. CONCLUSIONS

The results obtained in the present doctoral thesis produced the following conclusions:

1. Oxidative stress induces the alteration of mitochondrial dynamics in hippocampal neurons.
2. Aβ oligomers affect mitochondrial dynamics via oxidative stress and other mechanisms.
3. The flavonoid quercetin is able to completely prevent the effect of oxidative stress on mitochondrial dynamics.
4. The flavonoid quercetin is able to partially prevent the effect of Aβ oligomers on mitochondrial dynamics.
5. The PPARγ agonist rosiglitazone is able to partially prevent the effect of Aβ oligomers on mitochondrial dynamics.
6. The PPARγ agonist INT131 improves the neural network and its complexity.
7. The PPARγ agonist INT131 improves neuronal viability against the toxicity produced by Aβ oligomers.
8. The PPARγ agonist INT131 is able to significantly decrease the effect of Aβ oligomers on mitochondrial dynamics.

VIII. Other works produced during this thesis.

8.1. Amyloid-Peptide Nitrotyrosination Stabilizes Oligomers and Enhances NMDAR-Mediated Toxicity.

In this article, our objective was to study the nitrotyrosination of the Aβ peptide as a post-translational modification that increases synaptotoxicity.

Guivernau B, Bonet J, Valls-Comamala V, Bosch-Morató M, Godoy JA, Inestrosa NC, et al. Amyloid-β Peptide Nitrotyrosination Stabilizes Oligomers and Enhances NMDAR-Mediated Toxicity. J Neurosci. 2016 Nov 16;36(46):11693–703. DOI: 10.1523/JNEUROSCI.1081-16.2016

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