The physiopathological role of nitric oxide in the brain: from translational regulation of the GluN2B subunit to post-translational modifications of albumin in Alzheimer's disease

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hecho posible

La única manera de conocer los límites de lo posible es aventurarse
un poco más allá de ellos, hacia lo imposible…

Arthur Clarke

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Abstract

Nitric oxide (NO) is a molecule that has pleiotropic effects in brain and vascular system. Physiologically, NO induces the translation of the GluN2B subunit of N-methyl D-aspartate receptor (NMDARc) by derepressing it 5’untranslated region (5’UTR) effect. This pathway is due to the activation of the heme regulated eIF2α (HRI) kinase and prevents an excess of GluN2B levels, especially at the extrasynaptic areas, where it can trigger excitotoxicity. Pathologically, NO in a pro-oxidant environment such as in Alzheimer’s Disease (AD) reacts with superoxide anion producing peroxynitrite, which can nitrotyrosinates proteins. There are other concomitant oxidative processes that affect AD patient like protein glycation. Therefore the albumin, the most abundant plasmatic protein, in AD patients is more nitrotyrosinated and glycated, which affects its structure. Modified albumin has a reduced ability as an osmolarity buffer and it is hardly uptaken by hepatoma cells. Moreover, modified albumin binds more Aβ, contributing to maintain higher amount of amyloid in brain and plasma.

Resumen

El óxido nítrico (NO) es una molécula con efectos pleyotrópicos en cerebro y sistema vascular. Fisiológicamente, induce la traducción de la subunidad GluN2B del *N-methyl D-aspartate receptor* (NMDARc) al revertir la represión de su *5’untranslated region* (5’UTR). Este efecto se debe a la activación de la *heme regulated eIF2α kinase* (HRI) y previene el exceso de GluN2B, especialmente en regiones extrasinápticas, donde desencadena excitotoxicidad. Patológicamente, el NO en un ambiente pro-oxidatvio como el dado en la enfermedad de Alzheimer (AD) reacciona con el anión superóxido produciendo peroxinitrito, y causando entre otros efectos la nitrotirosinación de proteínas. Simultaneamente, las proteínas de pacientes con AD padecen otros procesos oxidativos como la glicación. Por tanto, la albúmina, la proteína plasmática más abundante, en estos pacientes está más nitrotirosinada y glicada, afectando su estructura. La albúmina modificada presenta menos capacidad para tamponar la osmolaridad y apenas es digerida por las células de hepatoma. Además, une más Aβ, contribuyendo a mantener más alta la carga amiloidogénica en cerebro y plasma.

Prologue

Nitric oxide (NO) is the main retrograde neurotransmitter as well as the major vasodilatant agent. In the glutamatergic synapses it is well-known that NO stimulates the soluble guanylate cyclase (sGC) increasing cyclic guanosine-3',5'-monophosphate (cGMP), which yields to a glutamate (Glut) release from the presynaptic ending forming an activation loop termed long term potentiation (LTP).This is the main mechanism for plasticity processes like memory and learning and it allows to store information and to recovery it. N-methyl D-Aspartate Receptors (NMDARc) are key players in memory processes. NMDARc are coincident detectors that need two different stimuli to get actived: the depolarization of the cell by sodium (Na^{+}) entry and the binding of the Glut and the coagonist glycine (Gly). These allow the magnesium (Mg^{2+}) release from the channels' pore and the entry of calcium ions (Ca^{2+}) through it. Ca^{2+} is a second messenger that triggers many intracellular pathways. By binding to calmodulin (CaM) it stimulates the neuronal nitric oxide synthase (nNOS), producing NO that will stimulate the Glut release by the presynaptic ending. In this thesis we report another effect of NO in the glutamatergic signalling but focused in the postsynaptic endings. The NO binds to the heme group of the heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI) producing its activation. Then HRI phosphorylates the eIF2α. The phosphorylation of eIF2α is a mechanism to avoid the translation of normal messenger ribonucleic acid (mRNAs) in situations of stress. But in proteins with a long 5' untranslated region (5'UTR) containing more than one upstream

AUG (uAUG) p-eIF2α enhances the translation of its mRNA. This system of regulation evade an excessive consume of energy in stress conditions, translating only the essential proteins. GluN2B belongs to these kinds of proteins and its mRNA is present in the synaptic spines waiting to be translated when its expression is necessary. GluN2B subunit is abundant in immature neurons where the synaptic spines are growing and plasticity events are predominant but when they mature is chiefly exchanged to GluN2A. However in mature neurons GluN2B is also present in the synapses and in the extrasynaptic areas where it can form active channels associated with GluN1 subunit. These active channels are functional and allow the Ca^{2+} entry and NO production, maintaining the neuronal communication.

Despite the effect described previously, NO can also be involved in a pathological process when it is produced in a pro-oxidant environment, like in Alzheimer disease (AD). AD is the most common dementia in elderly, and its prevalence increases with life expectancy. Nowadays it has turned into a capital problem due to the higher presence of aging people in developed countries. For this reason we need to understand the pathological processes occurring in AD. The beta-amyloid peptide (Aβ) has been described as the main effector in AD. Aβ is produced physiologically but in AD it is overproduced and less degradated. In this situation, Aβ is misfolded and aggregates extraneuronally producing superoxide anion ($O_2^{\cdot-}$). When this $O_2^{\cdot-}$ is combined with NO it triggers the peroxynitrite ($ONOO^-$) production. $ONOO^-$ damages proteins by nitrating the tyrosine residues, impairing its physiological

functions. AD is characterized by an increase of reactive oxygen species (ROS), exacerbating nitrotyrosination and glycation reactions. Albumin is a key protein in both the cerebrospinal fluid (CSF) and the plasma where it plays different functions as a buffering agent for osmolarity and free radicals, transport and trophic activities. The modification of albumin in both compartments, brain and blood, will affect dramatically to its functions and we have found that albumin is highly nitrotyrosinated and glycated in the brain and the blood of AD patients.

Abbreviations

AD- Alzheimer's disease

ADAM- a disintegrin and metalloprotease

AGE- advanced glycation end-products

AMPARc- α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors

APH-1-anterior pharynx defective 1

APLP1 or 2- APP-like protein 1 or 2

ApoE- apolipoprotein E

APP- amyloid precursor protein

Aβ- amyloid β protein

BACE-1- β -site APP cleaving enzyme 1

BBB-blood brain barrier

BSA- bovine serum albumin

CAA- cerebral amyloid angiopathy

CaM-calmodulin

CaMKII – Ca^{2+}/calmodulin-dependent protein kinase II

C83- C-terminal fragment 83

C99- C-terminal fragment 99

cAMP- cyclic adenosine monophosphate

cGMP- cyclic guanosine-3',5'-monophosphate

CNS- central nervous system

CREB- cAMP response element-binding

CSF-cerebral spinal fluid

DAHK- copper-chelating tetrapeptide aspartate-alanine-histidine-lysine

eIF2α- eukaryotic initiation factor 2 alpha

EPSP- excitatory postsynaptic potential

ERK- extracellular signal-regulated kinases

FAD- familial Alzheimer's disease

GABA-γ-aminobutyric acid

GC- guanylate cyclase

GCN2- general control non-derepressible 2 kinase

GluN2BRc - receptors bearing GluN2B subunits

GSH-glutathione

GTP- guanosine-5'-triphosphate

HSA-human serum albumin

HRI- heme-regulated eIF2α kinase

ICH-intracerebral hemorrhage

iNOS- inducible NOS

JNK- c-Jun N-terminal kinases

KO-knock out

LRP-1-low-density lipoprotein receptor-related protein-1

LTD- long term depression

LTP- long term potentiation

MAPK-mitogen activated protein kinases

MEK- MAPK of extracellular signal-regulated kinases

mGluR- metabotropic glutamate receptors

MG-methylglyoxal

mRNA- messenger ribonucleic acid

NADPH-nicotinamide-adenin-dinucleotide-phosphate

NFT-Neurofibrillary tangles

NMDA-N-methyl D-aspartate

NMDARc- NMDA receptor

NO-nitric oxide

NOS-nitric oxide synthases

nNOS- neuronal NOS

NT-HBD- N-terminal heme-binding domain

PEN2-presenilin enhancer 2

PERK-double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase

PI3K- phosphatidylinositol 3-kinase

PKC-protein kinase C

PKR-double stranded RNA-activated protein kinase

PP1-protein phosphatase1

PS- presenilin

PSD- postsynaptic density

RAGE-Receptor for Advanced Glycation End-products

ROS- reactive oxygen species

SAD- sporadic Alzheimer's disease

SAP-102 - synapse associated protein-102

sAPPα/β- soluble APPα/β

sGC- soluble guanylate cyclase

SOD-superoxide dismutase

STEP- Striatal -enriched protein Tyr phosphatase

TPI- triosephosphate isomerase

uAUG- upstream AUG

5'UTR- 5' untranslated region

VSMC- vascular smooth muscle cell

Molecular formulas

$^1O_2$- singlet oxygen

Ca^{2+}-calcium

Cu^{2+}-copper

$Fe^{2+/3+}$- iron

H_2O_2- hydrogen peroxide

K^+-potassium

Mg^{2+} - magnesium

Na^+-sodium

NO^+- nitrosonium ion

NO_2^- - nitrites

NO_2 –nitro group

$NO_2^{\cdot}$ -nitrite radical

NO_3^- - nitrates

O- atomic oxygen

$O_2^{\cdot -}$- superoxide anion

O_2-oxygen

O_3- ozone

$OH^{\cdot}$-hydroxyl radical

$ONOO^-$ - peroxynitrite

$ONOOCO_2^-$ -nitrosoperoxycarbonat

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I. INTRODUCTION

1. **AD and Aβ**

In 1907 Alois Alzheimer published an article termed “Über eine eigenartige Erkankung der Hirnrinde” [1, 2] describing a 51-year-old woman case who developed a rapid loss of memory combined with temporal and spatial disorientation, cognitive impairment, delirious and auditory hallucinations. The post-mortem autopsy revealed an atrophic brain with intracellular fibrils and thick bundles at the surface of the cells, distributed all over the cortex. This brain had neuron disintegration affecting also the glia and the endothelium. The increasing number of similar cases made necessary its classification as a specific illness, and in 1910, at the suggestion of Kraeplin, Alzheimer’s mentor, this pre-senile dementia was named Alzheimer’s Disease (AD) [3]. Despite Auguste D, Alzheimer’s patient, did not have a classical AD, nowadays AD is still diagnosed by the presence of two histopathological hallmarks: senile plaques and neurofibrillary tangles (NFT).

Senile plaques are formed by insoluble aggregates of Aβ [4, 5], a peptide released by the cleavage of the amyloid precursor protein (APP). These aggregates are flanked by morphologically altered neurons bearing NFT, which consists of abnormally aggregates of the *tau* microtubule-associated protein in a hyperphosphorylated state [6]. Aβ also aggregate in the brain vessel producing cerebral amyloid angiopathy (CAA) in more than 80% of the AD cases[7].

At present, AD is recognized like the most common dementia in the elderly, affecting more than 30 million individuals worldwide. In previous stages, patient shows severe memory deficits and a cognitive decline due to the neuronal damage at the hippocampal

formation. These stages are diagnosed as Middle Cognitive Impairment. As the disease progresses, neuronal death extends to the prefrontal cortex affecting severely the speech and the analytic abilities. Therefore the symptoms get progressively worse over 5 to 10 years.

AD can be classified as familiar (FAD) or sporadic (SAD), depending on the onset time of the symptoms. FAD appears before 65 years and accounts for less than 3% of all AD cases. The causes leading of FAD are unknown except for a 5% of the patients that shows mutations in APP or in presenilin (PS1, PS2) genes [8]. Despite these AD form, the most common form is SAD and occurs after 65 years old. The underlying cause of SAD is still to be determined. The principal risk factor for developing SAD is age, which doubles the incidence of the disease every 5 years after 65 years of age [9]. But even though aging is a risk factor, it can not explain the cognitive impairment and the appearance of SAD by itself. It is known that there are some factors associated to an increase in AD susceptibility, as the ε4 allele of the apolipoprotein E (ApoE)[10, 11], the second major risk factor for AD, or some polymorphisms such as P86L in CALHM1 [12].

1.1. The origin of Aβ deposition

The deposition of Aβ in brain and vessels could be due to an Aβ increase because of a higher production or a defective degradation. Neurons and vascular cells can produce Aβ, but they are not the only ones, and almost all cells in the body can do it [13-15]. In addition, there is an Aβ flux from the brain to the systemic

circulation but also in the opposite direction through the blood brain barrier (BBB) being the former enhanced under pathological conditions. Due to its amphipatic nature, to cross the BBB, Aβ needs specialized carriers and receptor transport mechanisms. These mechanisms control the uptake of circulating Aβ into the brain [16-22] and regulate it clearance [23-28]. The receptors involved, are the receptor for advanced glycation end-products (RAGE) and low-density lipoprotein receptor-related protein-1 (LRP1). RAGE receptors participates in brain uptake of free Aβ from the plasma [29], and, LRP1 mediates the Aβ clearance from brain to circulation [30] (**Fig. 1**). Opposite to the theory of the contribution of soluble plasmatic Aβ to brain deposition there is the fact that transgenic models with increased concentration of soluble Aβ in plasma have no brain lesions[31]. Furthermore, there is no evidence of increased Aβ production in sporadic CAA, so the defective Aβ degradation should be playing a key role. Neprilysin, acylpeptide hydrolase, endothelin-converting enzyme, insulin-degrading enzyme, beta-amyloid-converting enzyme 1, plasmin and matrix metalloproteases are the main enzymes involved in Aβ catabolic pathways [32-35]. Consistently, murine models with deletion of the genes codifying for these enzymes cause an increase of Aβ deposition[36, 37].

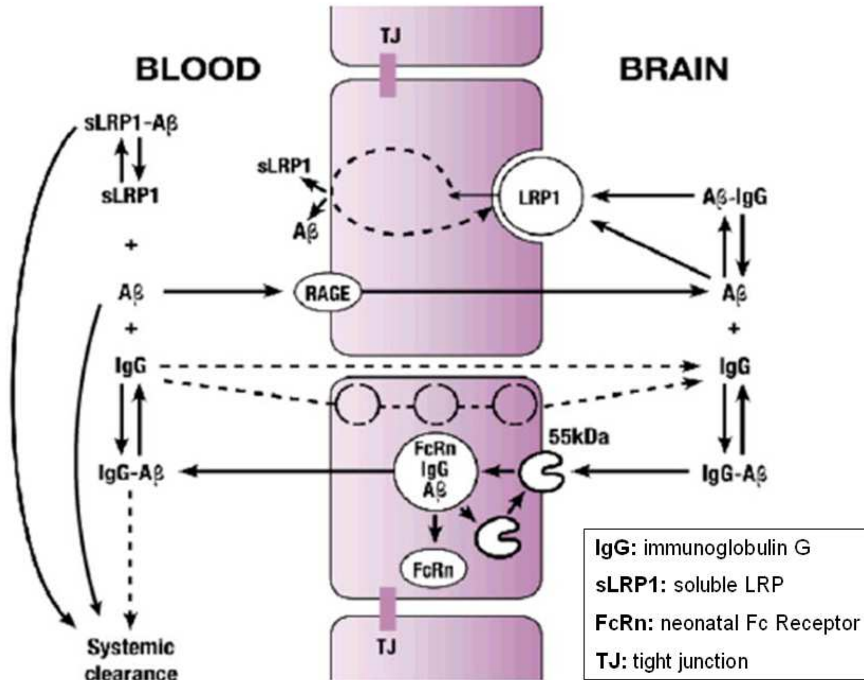


Figure 1. ***Aβ crossing through BBB***

Aβ is transported through BBB (in purple) by RAGE from blood to brain, and by LRP1 from brain to blood. The FcRn is also able to transport Aβ from brain when it is bound to IgG. (Figure extracted from Deane et al. 2009[38])

1.2. CAA and Aβ

CAA is a disorder characterized by the deposition of different types of amyloidogenic proteins. It occurs in the walls of leptomeningeal and cortical arteries, arterioles and less often in capillaries and veins of the central nervous system (CNS), and it induces the degeneration of the brain vessels [39]. Vascular Aβ deposition in CNS was first described by Gustav Oppenheim in 1909 [40], but it was not until 1938 that cerebral vascular abnormalities were recognized as CAA[41]. In 1954 Stefanos Pantelakis described their main

pathological features: preferential involvement of the small arteries and capillaries of the meninges, cerebral and cerebellar cortex, topographical distribution favouring the posterior brain regions, lack of staining in the white matter vessels, association with increased age and dementia and lack of association with hypertension, arteriosclerosis or the amyloidosis of the other organs[42].

CAA is a frequent clinical entity in aging people, being present in 10% to 40% of elderly brains and 80% or more of AD patients [43]. The classification depends on the type of amyloidogenic proteins implied: Aβ, amyloid-British protein, amyloid-Danish protein, cystain C, gelsolin, prion protein or transthyretin (TTR). The most common type is caused by Aβ and occurs in hereditary and sporadic forms. The familial forms are rare and occur in younger patients. This form that is associated to more severe clinical manifestations, appears due to mutations in the amyloid sequence as the Dutch, Flemish and Italian variants [44-50].

The risk of developing sporadic CAA is associated with ApoE ε4 and ε2 alleles [51]. Interestingly ApoE ε2, which exerts a protective effect on AD risk, in CAA increases the risk of intracerebral haemorrhage (ICH) [52, 53], probably related with the involvement of ApoE ε2 in the cardiovascular disease [54].

The main amyloid specie in the artery walls in CAA is the $A\beta_{1-40}$, which is also the most soluble form[55, 56]. The origin of this $A\beta_{1-40}$ is controversial: originally it was proposed to be produced by the smooth muscle cells of the vascular tunica media[57] but the existence of CAA in AD brain capillaries, that do not have vascular smooth

muscle cells, and in brain vessels from transgenic mouse overexpressing neuronal human-APP [58-61] indicates that the major source for vascular deposits are neurons. The Aβ produced by neurons is drained along the perivascular interstitial fluid pathways of the brain parenchyma and leptomeninges, depositing it along the vessels under specific pathologic conditions [62, 63]. However, a contribution to CAA by vascular smooth muscle cells [64] or other vascular cells [65] can not be discarded since they can produce $A\beta_{1-40}$ and $A\beta_{1-42}$. The Aβ uptake from plasma due to RAGE binding [66] could be also contributing to CAA development.

CAA can be completely asymptomatic, but amyloid deposition can weaken cerebral blood vessels walls, causing rupture and therefore leading to both asymptomatic microbleeds and ICH. CAA-related ICH accounts for 5-20% of all spontaneous ICH in elderly subjects. Amyloid deposits can also obliterate the vessel lumen, leading to ischemia and related clinical manifestations such as cerebral infarction, incomplete infarction and leukoaraisosis. It can produce neurological deficits, disturbances of consciousness, progressive cognitive decline dementia, and neuronal death as a consequence of the degeneration of cells in the walls of blood vessels [67]. It also causes the impairment of vasoactivity and the stimulation of proteolytic mechanisms, such as fibrinolysis, anticoagulation, and degradation of the extracellular matrix [68].

1.3. The APP metabolism

1.3.1. The APP physiological role

APP is a type-I transmembrane protein codified by a gene located on chromosome 21 in humans. Three isoforms have been described: those having 751 and 770 aminoacids, which are present in non neuronal cells and low expressed in neurons, and the isoform with 695 aminoacids, which is highly expressed in neurons [69].

APP belongs to a family of proteins that includes APP-like protein 1 (APLP1) and 2 (APLP2). All of they are processed in a similar way, although Aβ domain is unique to the APP protein. The APP knock out (KO) mouse is viable and fertile, showing a relatively subtle abnormal phenotype, including reduced body and brain size, impaired learning and LTP, reduced grip strength, hypersensitivity to seizures and increased frequency of corpus callous digenesis. These deleterious effects were mostly observed in early postnatal development [70]. Several studies suggest that APP homologues have some functional redundancy; only the APP/APLP1 double null mouse is viable [71], having APLP2 a crucial role when either APP or APLP1 is absent.

Roles for APP have been suggested in neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion and Ca^{2+} metabolism. Due to the similarity in topology and proteolytic processing between APP and Notch, a transmembrane receptor, it is suggested that APP may function as a membrane receptor, although the signalling events triggered by binding with some identified

ligands (Aβ, F-spondin and nectrin-1) [72-74] remains to be clarified. Moreover it is difficult to discern if all these effects were performed by the full length APP or its cleavage products.

1.3.2 APP cleavage: the secretases

APP is cleaved by different enzymes called secretases. There are three identified secretases: α, β and γ (**Fig. 2**).

1.3.2.1. The α secretase activity

The α-secretase is the only one not involved in the amyloidogenic pathway of the APP cleavage, and its activity is generally attributed to a disintegrin and metalloprotease (ADAM) family of proteases. Among this family ADAM 9, 10, 17 and even the 19 have shown to exert α-secretase activity [75-77]. Despite this fact ADAM 10 is the most active in brain [78-81].

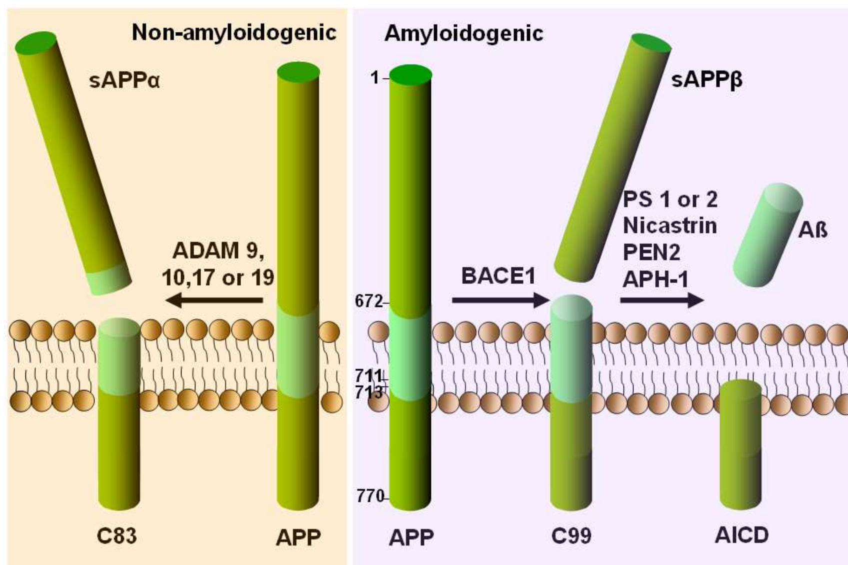


Figure 2. *APP cleavage by secretases*

The cleavage of APP by α-secretase produces the release of the soluble APPα (sAPPα) and the C-terminal fragment 83 (C83). The former fragment can be processed by the γ-secretase generating the p3 product, whose physiological role has not yet been established. The α-cleavage is the main cut of the APP located in plasma membrane [82] but there is also α-secretase activity over the APP in the trans-Golgi network, location where it competes with the β-secretase [83].

The APP processing by α-secretases is thought to be neuroprotective, since the sAPPα has demonstrated to protect cultured neurons against oxygen-glucose deprivation and excitotoxicity [84, 85]. A role to promote outgrowth and synaptogenesis [86, 87] has been proposed since *in vitro* studies demonstrated that its administration intracerebroventricularly enhanced learning and memory in mice and rats [88, 89]. Moreover, a study performed in an AD mouse model, showed that the overexpression of ADAM10 reduced Aβ production and plaque deposition in addition to present less cognitive deficits[90]. This would be due to the fact that an increase in APP processing by α-secretase, reduced the consequent β-cleavage and the Aβ production.

1.3.2.2. The β secretase activity

The β cleavage of APP produces sAPPβ and the C-terminal fragment 99 (C99), from which γ-secretase renders Aβ. β-site APP cleaving enzyme 1 (BACE1), although it is not the only β-secretase in the brain, it is the most relevant one [91]. In fact, deficient BACE1

mice show a lack of β-secretase activity and Aβ formation in neurons [92-94]. Many studies have demonstrated the BACE1 contribution to AD since its expression and activity is increased in brains of AD patients [95, 96]. Despite its pathological role, BACE1 also has a physiological function since BACE1 KO shows severe hypo-myelination of peripheral nerves, modest in the CNS [97], reduction in spine density in hippocampal pyramidal neurons and alteration in behaviour tests related with schizophrenia, as well as impairments in cognition and social recognition [98].

The APP cutting by BACE1, which mainly occurs in endocytic vesicles, has been proposed to take place during neuronal depolarization, relocalizing APP to the BACE1-containing membrane microdomains [99]. The sAPPβ produced, do not share the neuroprotective properties of sAPPα [100], and is critically involved in the pruning of synapses during development of central and peripheral neurons and acts as a ligand for death receptor 6, which produces axonal disintegration [101].

1.3.2.3. The γ-secretase activity

The γ-secretase enzymes cuts the C-terminal fragments C83 and C99 produced in the previous cleavages by α- or β-secretases, respectively. In order to be active, the γ-secretase needs the formation of a four protein complex containing: PS1 and 2, Nicastrin, anterior pharynx defective 1 (APH-1) and presenilin enhancer 2 (PEN2) [102]. PS are the catalytic subunits responsible for the intramembrane aspartyl protease activity [103]. They can produce different Aβ species, being $A\beta_{1-40}$ and $A\beta_{1-42}$ the predominant. The

higher production is for $A\beta_{1-40}$ but $A\beta_{1-42}$ is more prone to aggregate and can act as a seed for $A\beta_{1-40}$ aggregation [104, 105]. Therefore PS function determines $A\beta_{1-42}/_{1-40}$ ratio influencing the deposition of Aβ [106]. In addition, the mutation in PS genes is associated with FAD, determining the functional importance of the γ-secretase complex in AD. However, results obtained in conditional KO studies suggest that reduced activity of γ-secretase is associated with detrimental side effects in the brain, especially in older animals [107, 108]. Regarding these observations and considering that the γ-secretase also has other substrates such as N-cadherin, Notch or LRP, discards its inhibition as a therapeutic approach in AD for its important implication in surface receptor-linked signalling pathways.

1.3.3. Aβ and its toxicity

Aβ is the most relevant protein in the amyloid hypothesis of AD. Aβ is generated in the endosomal compartment by the concerted action of β- and γ-secretases [109-112], and is subsequently secreted to the extracellular space through exocytosis, where its aggregation is observed by the change to a β-sheet structure. Intracellular Aβ has been observed as well, both in animal models of AD and human patients [113, 114], but this could be due to the normal trafficking of Aβ or to an abnormal uptake from the extracellular space.

There are some reasons to support that the amyloid accumulation triggers AD. One of them is that the trisomy of the chromosome 21, where APP is located, causes AD type dementia [115]. Furthermore, Aβ production is either increased in most hereditary cases and

animal models of AD or the ratio of Aβ$_{1-42}$ to Aβ$_{1-40}$ is higher. This fact is related with the causative AD mutations in PS genes, because it can shift the Aβ processing to Aβ$_{1-42}$, the more aggregogenic form. There are also APP mutations that make Aβ more prone to aggregate such as the Dutch and Artic mutations[116-118], and others that enhance the β-secretase cleavage, increasing the Aβ production such as the Swedish mutation[119].

Aβ can assembly forming oligomers (from two to eight Aβ units), protofibrils and fibrils, the biggest aggregates. Although fibrils are found in the senile plaques, it has been reported that oligomers are the most toxic form [120-125]. In a variety of cell culture models, Aβ has shown to cause toxicity to neurons or inducing dendritic spine loss and LTP impairment [126-132]. The mechanism involved in these effects seems to be related to the oxidative stress[133-135].

Aβ fibrils induce free radical production [136, 137] mediating Aβ cytotoxicity. In agreement there are evidences that show that micromolar concentrations of Aβ increase hydrogen peroxide (H_2O_2) in culture. Besides treatments with antioxidants like catalase and superoxide dismutase (SOD) prevents its toxicity [138]. Furthermore intracellular ROS are increased in Down's syndrome patients who *per se* overexpress APP [139].

On the other hand soluble oligomers can inhibit N-methyl D-aspartate (NMDA)-mediated synaptic transmission causing spine and synapse loss [140]. Studies using extracts from brains of AD patients and hippocampal slice cultures show that Aβ dimers are the most potent form of Aβ oligomers, and they are able to inhibit NMDA mediated synaptic transmission [141]. Higher molecular

weight oligomers and insoluble aggregates are also able to release Aβ dimers [142]. Moreover Aβ dimers isolated from AD brains can induce *tau* hyperphosphorilation in hippocampal cultures correlating with neurite cytoskeleton disruption.
Despite Aβ aggregates was thought to be the main toxic form of Aβ, it is currently known that the soluble oligomers are the most noxious components, suggesting that the invariant accumulation of insoluble Aβ in plaques may be a protective mechanism by storing Aβ [143, 144].

2. The NO

NO is a gaseous molecule extraordinarily labile, with a half-life of only about 3-5 seconds [145]. Albeit its production is mainly due to NO synthases (NOS) it can also be synthesized by the reaction of H_2O_2 and D-or L-Arg in a non-enzymatic way [146] and by the reduction of nitrites (NO_2^-) in acid and reducing conditions, as occurs in the ischemic process [147]. There are four NOS: nNOS, endothelial NOS, inducible (iNOS) and mitochondrial NOS. All of them, except the iNOS are Ca^{2+}-CaM dependent enzymes that generates NO that only lasts a few minutes. Instead of this, the iNOS regulation depends on the *novo* synthesis [148] under immunological or inflammatory stimuli, and produces NO that lasts hours or days [149].
To produce NO, NOS uses L-Arg as a substrate in the presence of nicotinamide-adenin-dinucleotide-phosphate (NADPH) and oxygen (O_2) (**Fig 3**). L-Arg can be synthesized from Glut [150] or by recycling citrulline [151], the other product of NOS.

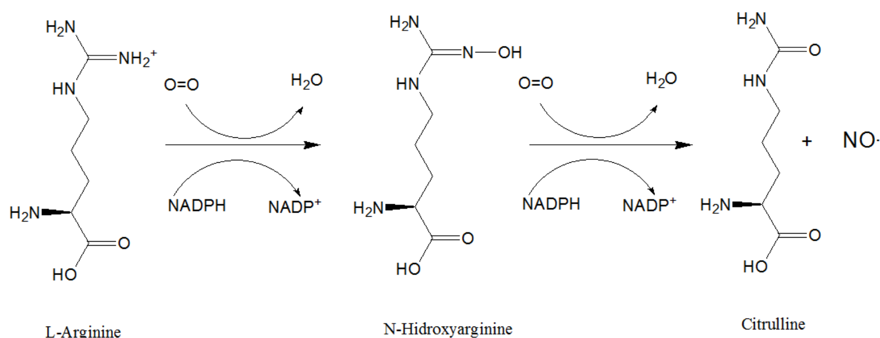


Figure 3. ***NO synthesis by NOS***

NO is a molecule with an unpaired electron (**Fig.4**), making it a free radical, that can also exist as a nitrosonium ion (NO^+) depending on the cellular redox status [152]. Thus, NO is thermodynamically unstable and tends to react with other molecules producing NO_2^-, nitrates (NO_3^-), and $ONOO^-$.

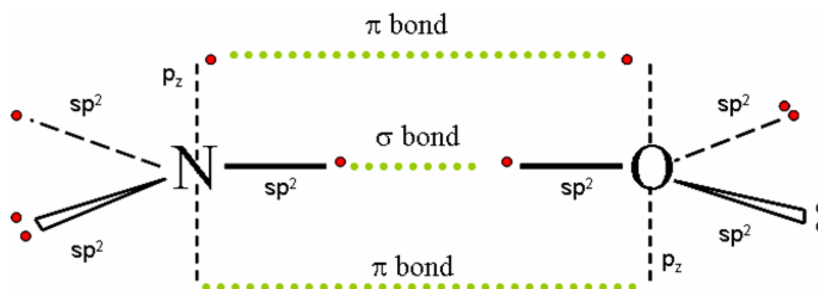


Figure 4. ***Molecular structure of NO***

Red dots are valence electrons; green dotted line represent molecular bonds; sp^2: orbitals of linear combination from atomic orbitals 2s, $2p_x$ and $2p_y$.

2.1. Reactive nitrogen species

The final product molecules of the NO reactions have different effects: $ONOO^-$ can trigger protein nitrotyrosination [153, 154]; ONOOH, hydroxyl anion ($OH^\cdot$) and nitrite radical ($NO_2^\cdot$) act as oxidant agents, and its NO^+ form produces protein nitrosylation.

Nitrotyrosination, the addition of a nitro (NO_2) group into a Tyr, may alters the normal protein activity leading to a loss of function of proteins such are the mitochondrial MnSOD [155, 156], actin [157], glutamine synthase [158], heme oxygenases [159], iron regulatory protein-1 [160], histone deacetylase 2 [161], mammal aldolase A [162], p53 [163] and prostacyclin synthase [164].

S-nitrosylation, which consists in the incorporation of a NO moiety into a thiol group, can decrease the activity of the target enzymes, and may lead to activate matrix metalloproteases, which pathological process is reported in stroke and neurodegenerative disease [165, 166]. Despite these effects S-nitrosylation has been also proposed as a mechanism to store NO as producing s-nitrosothiols in albumin [167]. This effect can act as neural protector because of the formation of the antioxidant S-nitroso-L-glutathione [168] at the same time that it prevents $ONOO^-$ production by substrate competition[169].

2.2. NO signalling pathways

NO is involved in several signalling pathways. Among them its implication in the activation of guanylate cyclase (GC) is the best well-known due to its relevance in vascular smooth muscle cell (VSMC) relaxation. GC catalyzes the change of guanosine-5'-triphosphate (GTP) into cGMP [170]. The cGMP is a second

messenger that activates protein kinases responsibles of the intracellular Ca^{2+} and anions flux control [171, 172]. cGMP can also increase the cyclic adenosine monophosphate (cAMP) intracellular levels through the inhibition of the phosphodiesterase III [173] or decreasing them by stimulating the phosphodiesterase II [174].

In relation with the mitochondrial respiratory chain, NO competes with oxygen for the cytochrome c oxidase. NO inhibits this enzyme reversibly and decreases oxygen consumption [175, 176]. Likewise, it promotes the mitochondrial biogenesis, regulating the cellular energetic metabolism[177, 178].

NO has an inhibitory role regarding cell proliferation [179, 180]. At first the increase in the activity of the NOS would produce a shift of L-Arg metabolism to NO generation, decreasing the production of L-ornitine [181], a substrate for polyamine biogenesis. On the other hand NO activates Ras signalling pathway [182], S-nitrosylates several transcription factors [183], inhibits cyclin A, and activates the cyclin-dependent kinase inhibitor $p21^{Cip1}$ [184]. Since differentiation is closely coupled with cessation of proliferation, these mechanisms would be involved in neuronal differentiation promoted by NO[185].

NO can also affect the mitogen activated protein kinases (MAPK) intracellular signalling. It activates the *MAPK of extracellular signal-regulated kinases (ERK)* (MEK), ERK and c-Jun N terminal kinase (JNK) [186], and it also inhibits the platelet aggregation due to the prevention of phosphatidylinositol 3-kinase (PI3K) activation[187].

NO has a dual effect in apoptosis therefore it can be a pro-apoptotic o an anti-apoptotic molecule. In physiological conditions NO is considered anti-apoptotic due to its S-nitrosylation hability, its

inhibition of caspases [188, 189] and the release of Bax [190]. NO also induces cytoprotective genes as HSP70 [191] and inhibits cytochrome c release [192, 193], it maintains the anti-apoptotic Bcl-2 levels and inactivates the pro-apoptotic Bad and procaspase-9 by phosphorylation [194]. In pathophysiological processes, though, it action depends on the NO location, concentration and the co-existence with other agents. The pro-apoptotic role of NO is meditated by its binding to cytochrome c oxidase, inducing the formation of $O_2^{\cdot -}$ and generating $ONOO^-$ in the mitochondria [195]. NO may also induce apoptosis by activating the MAPK pathway, involved in stress response [196, 197]. Under this stress situation, NO will activate the caspase cascades by its effect in the increase of ceramide [198].

2.3. NO physiological functions

NO also plays fundamental roles in important physiological functions (**Fig.5**). Among them, NO is crucial in vasodilatation, immune response and neurotransmission in neurons. These functions are reviewed in the following paragraphs.

It is important to highlight that NO levels have to be tightly regulated as under physiological concentrations it modulates the previously mentioned responses; but at higher concentrations and oxidative stress conditions, NO can be harmful and contribute to ageing-associated diseases [199].

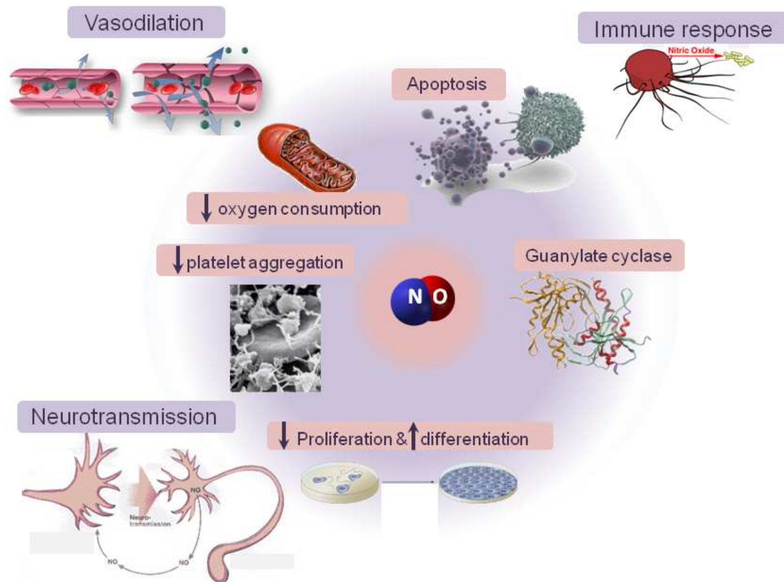


Figure 5. ***NO signalling pathways and effects***

2.3.1 Vascular effects

Endothelial cells produce NO that lead to VSMC relaxation via the generation of cGMP. It activates protein kinase G (PKG)-dependent ion channels inducing hyperpolarization [200, 201] or a direct activation of Ca^{2+}-dependent potassium (K^+) channels [202-207]. Moreover NO activates sarcoplasmic-endoplasmic reticulum Ca^{2+}ATPases, that deplete the Ca^{2+} levels of the cytosol [208]. On the other hand NO may also have another protective vascular role mediating the inhibition of VSMC proliferation and platelet aggregation [209].

2.3.2 Immune role

In leukocytes iNOS is expressed after immune or inflammatory stimulation, producing a large amount of NO lasting a long time, up to several days. Its role in the immune response [210] is due to the $ONOO^-$ formation, which is a powerful anti-microbial and anti-tumoral agent [211]. Besides, it has been reported that NO activates cyclooxygenase-II producing pro-inflammatory molecules in glial cells [212] and it is also a well-known regulator of leukocyte adhesion to vessels [213].

2.3.3. NO neuronal function

In the brain, Garthwaite was the first to observe that the activation of NMDARc leaded to a release of NO [214], playing a key role in synaptic plasticity [215]. NO has been also associated to pain perception [216], sleep control, appetite, thermoregulation [217] and neural development [218].

Hippocampus, striatum, hypothalamus and locus coeruleus are brain regions where it has been demonstrated that NMDARc stimulation activates nNOS[219-221] with a peak at 5-15 min, returning to basal levels after 60 min, most probably due to substrate exhaustion [222]. This mechanism is due to the quality of NO as a retrograde neurotransmitter, stimulating Glut release by the presynaptic terminal [223]. It also plays a key role in LTP, which is, as mentioned before, the main mechanism of information storage.

LTP consists in the continuous synaptic activation of specific routes of the brain. The postsynaptic activation is maintained by the NO-mediated Glut release in a cGMP-dependent pathway in the

presynaptic terminal [224]. Moreover NO has a protective role by blocking caspases [225] and regulating an excessive NMDARc activation by S-nitrosylation, thereby avoiding its toxic effects [226]. Nevertheless, NO also mediates NMDARc-dependent neurotoxicity [227] and inhibits the glutamine synthetase, a brain ammonia detoxifying enzyme, by nitration and nitrosylation [228].

On the other hand, NO produced by NMDARc, increase the release of acetylcholine [229, 230], noradrenaline [231, 232], serotonin [233, 234] and adenosine, a neuroprotectant neurotransmitter [235]. On its role on the γ-aminobutiric acid (GABA), NO has different effects depending on its concentrations. It inhibits GABA release at low basal levels, but it increases it release at high concentrations [236]. NO has the same dual effect regarding histamine. When NO leads to acetylcholine liberation it activates muscarinic receptors M1 inhibiting histamine release, but when M1 are blocked, the Glut released by NO produce the contrary effect[237].

2.3.4. NO and the translational control

2.3.4.1. The HRI

NO is an activator of the HRI[238]. HRI is a kinase that has been isolated from rabbit reticulocytes and also cloned from rat brain and presents two heme-binding sites: His119/120 in the N-terminal and Cys409 in the catalytic domain. NO is able to bind to the N-terminal heme-binding domain (NT-HBD), disrupting the inhibitory interaction between the NT-HBD and the catalytic domain, thus activating HRI [239]. Specifically, NO can reduce heme-Fe(III) to heme-Fe(II) [240], which binds NO forming a 5-coordinate heme-

Fe(II) NO complex, changing the HRI kinase conformation [241, 242]. This model might potentially be affected by the phosphorylation states of HRI and the allosteric effect of eIF2 binding, which can modify the heme-binding affinity and HRI conformation [243, 244].

2.3.4.2. The eIF2α

Four kinases are involved in the phosphorylation of the eIF2α: the HRI, the protein kinase RNA activated (PKR), the general control non-derepressible 2 kinase (GCN2) and the double-stranded RNA-activated protein kinase-like ER kinase (PERK) [245-247].

The eIF2α phosphorylation regulates the initiation step of mRNA translation [248-252] (**Fig.6**). This eIF2α factor is a G protein composed by three subunits: α, β and γ. The dephosphorylated form binds a transference mRNA coupled to methionine (Met-tRNA) and a GTP molecule. This molecular complex is known as the ternary complex that loads the initiating Met-tRNA to the 40S ribosome-mRNA complex. The ternary complex plus the ribosome conform the initiation machinery of translation and scans the 5' UTR of the mRNA looking for the main AUG [253]. Once the machinery reaches this AUG the translation begins. This process will consum the energy obtained from the conversion of a GTP molecule to a GDP. Only the complexes bound to GTP can perform the scanning, so the system needs to replace the consumed GDP for a new GTP in order to continue the other rounds of initiation. The guanine exchange factor eIF2B is responsible of doing it by interacting with eIF2α [254-256].

When the eIF2α is phosphorylated at the amino acid Ser51, it increases its affinity for eIF2B, thus, sequestering the latter in an inactive complex. This will lead to a lower or stopped translation initiation due to the lack of eIF2-GTP [257].

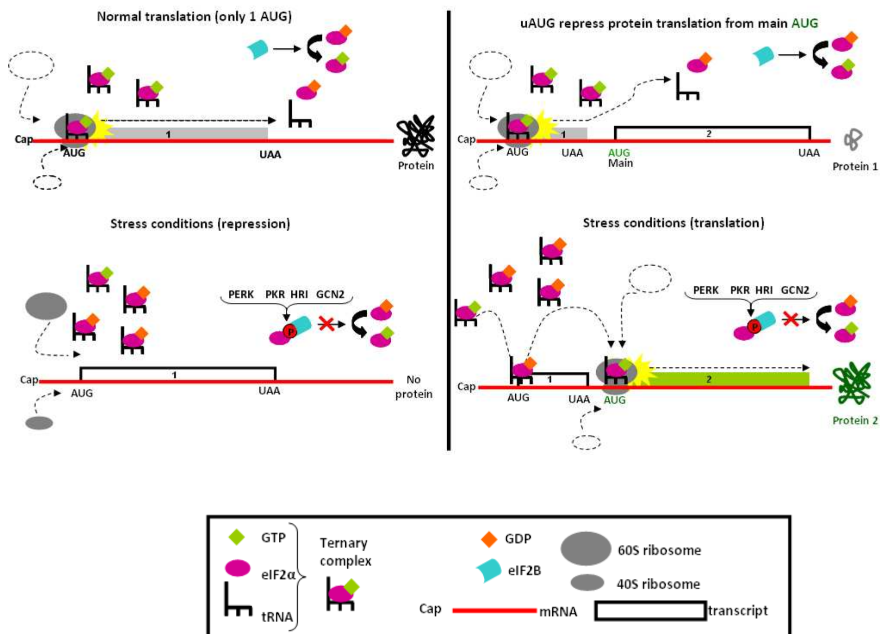


Figure 6. ***Normal and uAUG 5'UTR translation***

In normal conditions the ternary complex scans the 5'UTR looking for the main AUG. When a transcript has a 5'UTR with several AUG, the initiation translational machinery does not find the main AUG. Under stress conditions, the eIF2α is phosphorylated and the exchanger eIF2B binds to it, inhibiting the conversion of eIF2α-GDP to GTP by competitive binding. Then the eIF2α phosphorylation produces a reduced availability of active initiation complex and represses the translation of normal proteins. However this situation changes in proteins with several AUG in its 5'UTR, because the eIF2α phosphorylation makes more efficient its translation.

Physiologically, the arrest of protein synthesis by eIF2α phosphorylation is a compensatory mechanism to save energy under stress conditions, such as oxidative stress, nutrient deprivation, missfolded proteins, ER stress or viral infection. The above mentioned kinases that phosphorylate eIF2α are responsible for detecting these stimuli. Specifically, HRI detects the oxidative stress, the heme deficiency and the NO presence [258-261]. Nevertheless, there are proteins needed in stressful conditions and they will need to be translated during the global translation arrest. To ensure their synthesis under conditions of eIF2α phosphorylation, these genes have a specific system of translational regulation that allows its translation. This is mediated by the presence of AUG codons and GC rich content sequences within their long 5'UTR. When there are several uAUG in 5'UTR, the ribosome with the ternary complex can bind to uAUG and does not reach the main one [262, 263]. But when eIF2α is phosphorylated there is less amount of initiation complex in the active form, and the scanning of the ribosome is more effective, skipping inhibitory uAUGs and beginning the translation. Less of 10% of the eukaryotic mRNAs contain AUG codons within their 5'UTR, but it is not uncommon in genes with critical cellular roles, as in oncogenes and another genes involved in growth and differentiation [264-266]. Interestingly, the uAUG 5'UTR repression system was described for BACE1 [267-270] and it is reverted by eIF2α phosphorylation [271].

All NMDA subunits except for GluN2C can be included in this group of genes since they have several uAUG [272]. Specifically,

human GluN2B contains 3 uAUG in its 179 bp 5'UTR and the study of its regulation in physiological conditions, may help us understand what happens in pathologic situations like excitotoxicity in AD.

3. The glutamatergic transmission in brain

Most excitatory synapses use the neurotransmitter Glut, which can act through two types of Glut receptors (**Fig.7**), the ionotropic and the metabotropic receptors.

There are three ionotropic receptors: the NMDARc, the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARc) and the kaynate receptor. On the other hand the metabotropic Glut receptors (mGluR) are members of the Family C of G-protein-coupled receptors and are formed by eight subtypes, $mGluR_1$ to $mGluR_8$. The AMPARc and NMDARc are located in the region of the postsynaptic density where they respond to the Glut released in the synaptic cleft. However the mGluR tend to lie outside the synaptic cleft, thus responding to Glut that spills out of the cleft.

The NMDARc is a constant feature of the synapses but the AMPARc expression and insertion in the synaptic membrane is much more variable [273, 274]. This is thought to be due to the large cytosolic tail present in NMDARc (600 aminoacids or more) that could lead to multiple and stronger scaffold interactions than the AMPARc [275]. Nevertheless AMPARc trafficking and translocation into the postsynaptic membrane is a hallmark of synaptic plasticity and it is regulated by several proteins [276].

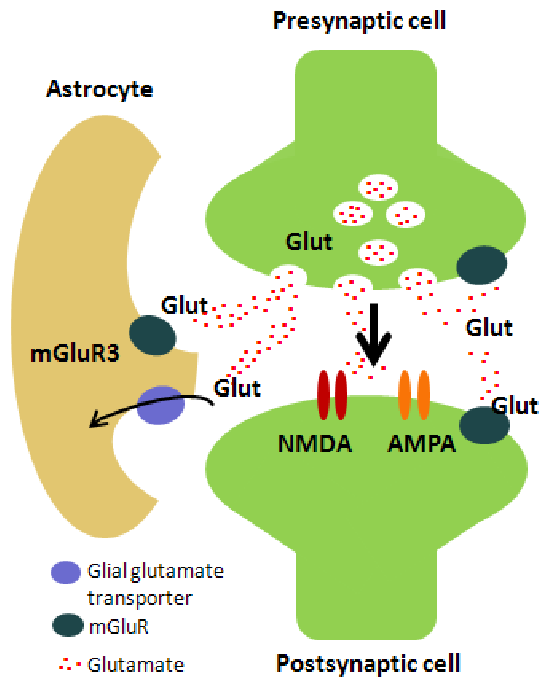


Figure 7. ***Glutamatergic transmission***

Representation of a synapse between a presynaptic and a postsynaptic neuron. An astrocyte surrounds it. The Glut released at the synaptic cleft stimulates the ionic receptors NMDA and AMPA, and the metrabotopic receptor mGluR. In contrast to AMPA or NMDA which are always in postsynaptical, mGluR are also present in astrocytes and in the presinaptic neurons. Adapted from Weinberger, 2007[277].

3.1. LTP and Glut

Neuron communication starts when the presynaptic neuron fires an action potential that propagates down the axon up to the synaptic ending. There, it provides the depolarizing signal to activate voltage-operated channels producing an inward current due to the entry of the Ca^{2+}. This will trigger the release of neurotransmitters by exocytosis. In the postsynaptic ending Glut activates different

receptors, such as the AMPARc allowing an inward flow of Na^+ and Ca^{2+}. As a result, an excitatory postsynaptic potential (EPSP) is generated. The EPSP facilitates the opening of NMDARc that enhances the depolarization by allowing a massive entry of Na^+ and Ca^{2+} into the dendrites. Every individual EPSP are summed to produce the global depolarization that fires an action potential in the postsynaptic neuron completing the flow of information between both neurons [278, 279].

Memory and learning are based on the modulation of neuronal connections by synaptic plasticity. The store of information and its recovery are produced as a balance between the reinforcement and depression of neuronal connections. It is achieved by either an intense stimulation or the integration of several stimuli by a coincident detector [280, 281] that triggers the LTP or the long-term depression (LTD). Such modifications can be long-lasting or changing along the time, therefore contributing to the different plastic events. NMDARc are thought to be a coincident detector since they respond to postsynaptic depolarization and to Glut from the presynaptic ending [282]. Thus NMDARc are playing a key role in LTP and LTD, hence in memory and learning.

3.2. The NMDARc

3.2.1. NMDARc structure

NMDARc are composed by different subunits: the ubiquitously expressed GluN1 subunit; a family of four distinct GluN2 subunits (A, B, C and D); and two GluN3 subunits [283-286]. Each subunit is

formed by 3 transmembrane segments and a re-entrant loop, with an N-terminal extracellular domain and a C-terminal intracellular domain. To be functional, the NMDARc assemblies in a tetramer complex composed by two GluN1 subunits and two GluN2. Occasionally also one GluN3 subunit may form part of the complex[287-291]. Both the GluN1 and GluN2 subunits contribute to the formation of the ion channel. The NMDARc is unique since the opening of the channels' pore requires the binding of two different agonists: Glut and Gly [292]. The Glut-binding site is on the GluN2 subunits, and the Gly-binding site is located on the GluN1 subunits.
The NMDARc is permeable to monovalent cations, including Na^+ and K^+, and divalent cations, most notably Ca^{2+}. However, there is a binding site within the channel pore for Mg^{2+} that blocks ion flow through the channel at resting membrane potential (**Fig. 8**). Mg^{2+} is expelled when membranes are depolarised. Therefore, both depolarisation of the postsynaptic neuron and presynaptic release of Glut are required for maximum current flow through the NMDARc.

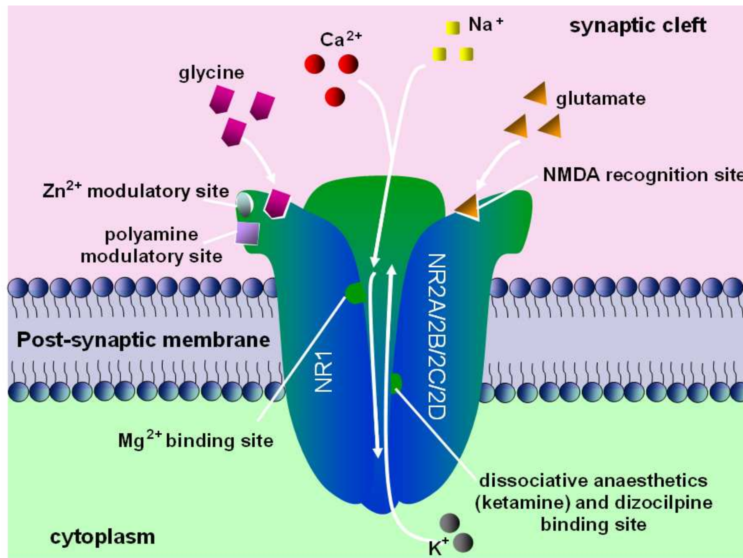


Figure 8. ***Structure of NMDARc***

Tetrameric NMDARc at the postsynaptic membrane. The GluN1 subunit has the binding site for Gly and the GluN2 the Glut binding site. The pore formed by the GluN1 and GluN2 is permeable to cations, especially Ca^{2+}. In the pore there is the Mg^{2+} binding site, which blocks the ionic flow until a depolarizating stimuli arrives.

3.2.2. NMDARc function

NMDARc allows the Ca^{2+} entry inside the cell and may activate beneficial or deleterious neuronal pathways. This ambiguous role of NMDARc is called the NMDA paradox. It is thought that the location of the NMDARc influences whether it is coupled to pro-death or pro-survival signals. According to this model, synaptic NMDARc are neuroprotective, whereas extrasynaptic NMDARc preferentially initiate cell death pathways [293].

During the brain development, synaptic NMDARc suffer a switch: the early expressed receptors bearing GluN2B subunits

(GluN2BRc) are replaced by GluN2A-containing receptors, which eventually predominates at the synapse [294-304]. In mature neurons, extrasynaptic domains are more often enriched inGluN2BRc, although they can also be found in synaptic areas [305-313]. GluN2 location is related to its preference to bind postsynaptic density (PSD) scaffold proteins. GluN2B has demonstrated to have a high affinity for synapse associated protein-102 (SAP-102) and it targets this subunit to the extrasynaptic zones, while PSD-93 and PSD-95 display higher affinities for the GluN2A subunit in enriched active sites [314]. Nevertheless they can move to synaptic areas from extrasynaptic locations [315] and it has been characterized that NMDARc with GluN2B subunits have a higher mobility in synaptic and extrasynatic compartments than those with GluN2A[316, 317].

GluN2BRc in extra-synaptic zones mediate apoptosis. It decreases GluN1/GluN2A active channels [318] and reduces the pro-survival gene transcription. Consequently, Glut excytotoxicity correlates more closely with increased levels of GluN2B rather than GluN2A subunits [319, 320]. The reason why GluN2B is thought to mediate excytotoxicity is because GluN1/GluN2B complex have longer current-decay times, which allows a greater Ca^{2+} influx [321].

Despite of its predominant expression in extrasynapses, GluN2B is also present in the synaptic zones where it interacts indirectly with the the nNOS via the scaffold protein PSD-95 [322]. The importance of GluN2B comes also from the fact that it is the most abundant GluN2 subunit in the hippocampus, an area that degenerates markedly in AD. Moreover, the distribution of both GluN2B and

GluN2A in brain correlates with the regional pattern affected in AD [323].

3.2.3. NMDARc in AD

Synaptic dysfunction and nerve-ending loss are more accurate predictors of the cognitive impairment than Aβ plaque load or NFT presence. These neurodegenerative hallmarks could be directly related with the over-stimulation of NMDARc inducing excytotoxicity [324]. Thus NMDA expression could be playing an important role in AD [325-327]. This is actually the rationale for the treatment with memantine, an uncompetitive NMDARc inhibitor [328], which slows the disease progression in subjects with moderate to severe AD [329-331].

Aβ, especially the oligomeric forms, could mediate its toxic effects through NMDARc inducing LTP disruption, correlating it with AD memory deficits. In fact Aβ oligomers increase intracellular Ca^{2+} levels through its effect on the GluN1 NMDARc subunit, leading a synaptic mitochondrial dysfunction and an excessive ROS formation [332]. Furthermore Aβ impairs both the early and the late phase of LTP. In the early phase, Aβ can stimulate the protein phosphatase 1 (PP1) [333] which may dephosphorylate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [334], impairing the phosphorylation of the GluR1 of the AMPARc [335], that normally occurs under LTP-inducing stimulus. In the late phase, Aβ enhances the dephosphorylation of cAMP response element-binding (CREB) through calcineurin [336], leading to a reduction of protein synthesis necessary in late phase of LTP [337].

The triggering of calcineurin pathway by Aβ oligomers, such as dimmers and trimers, can stimulate LTD instead of LTP [338], suggesting that the inhibition of LTP by Aβ oligomers reflects a shift in the LTP/LTD balance rather than a direct blockade of the LTP-inducing pathways [339].

Furthermore Aβ may increase the amount of Glut in synapses by inhibiting Glut re-uptake [340] and this spillover can activate extrasynaptic GluN2BRc [341-343].

3.2.3.1 GluN2B in Aβ toxicity

In the scenario of Aβ toxicity through NMDARc, GluN2BRc has been reported to play an essential role. The absence of GluN2BRc in KO mice lead to perinatal death whereas mice lacking GluN2A, GluN2C and GluN2D subtypes are viable [344-346]- and actually some studies propose that both increased and decreased GluNR2B activity may have deleterious effects. Hu in 2009 demonstrate that injection of selective GluN2B inhibitors, ifenprodil and Ro 25-6981, in rats, prevented the inhibition of LTP by soluble $A\beta_{1-42}$ [347]. This study demonstrates that Aβ renders an increase of tumor necrosis factor-α stimulating a higher availability of extracellular Glut that can acts on GluN2BRc. In addition, works ablating GluN2B in mice's principal neurons show moderate deficits in LTP [348] and the injection of $A\beta_{1-42}$ was unable to induce further deficits[349]. Nevertheless, the heterozygous GluN2B mice showed normal LTP and no $A\beta_{1-42}$ induced deficits [350]. However, the transfection of GluN1/GluN2A and GluN1/GluN2B in Xenopus

oocytes shows that Aβ activate particularly GluN2A receptors instead of GluN2B [351].

The phosphorylation of GluN2B subunits allows its expression on the cell surface, but the application of $A\beta_{1-42}$ in cortical neurons yield to GluN2B dephosphorylation and endocytosis. This effect was mediated through an α-7 nicotinic receptor. That activates PP2B leading to a dephosphorylation of the Striatal-enriched protein Tyr phosphatase (STEP); STEP61, which is the brain isoform increased in AD [352] is then active and dephosphorylates GluN2B [353]. Other studies demonstrate that there is a significantly lower expression of GluN2A and GluN2B in susceptible regions of the AD brain compared with the controls [354]. However, there are no differences in the reduction between the expression of GluN2A and GluN2B. This effect could be explained by the glutamatergic neurons death [355]. Taken all these evidences together, one can conclude that NMDA expression, and especially GluN2BRc, has to be tightly regulated because an overcome or a deficit of its expression can produce LTP impairment.

4. Nitro-oxidative stress in AD

The nitro-oxidative stress is the main toxic mechanism mediated by Aβ. In the following paragraphs the agents responsible for this toxicity are described as well as its dual physiopathological function.

4.1. Oxidative stress

4.1.1 Free radicals

A free radical is a molecule, neutral or with charge, which contains one or more unpaired electrons. The production of free radicals occurs by the addition or the loss of electrons by redox reactions during cellular metabolism. The reactivity of this chemical species comes from these electrons that tend to pair with electrons from other molecules.

Although free radicals are mostly harmful, at low concentrations they may play a role in different cellular functions such as activation of transcriptional factors, signal transduction or even triggering an immune response, since activated phagocytes produce $O_2^{\cdot -}$ by NADPH oxidase complex to kill bacteria. They can also act as messenger molecules in LTP and synaptic signalling, like $O_2^{\cdot -}$ which increase after NMDARc activation [356] or H_2O_2, which at low micromolar concentrations potentiates LTP induced by high frequency stimuli [357, 358].

4.1.2 ROS

Oxygen is an essential element for life but it can be toxic when it produces ROS. ROS are the products of disaggregation or excitation of O_2 like the atomic oxygen (O), ozone (O_3) and singlet oxygen ($^1O_2$). Other ROS are partially reduced like the H_2O_2, the $O_2^{\cdot -}$ and the $OH^{\cdot}$ [359]. $^1O_2$ is very reactive and can react with most of the cellular molecules [360]. O_2 and H_2O_2 are poor reactors but may generate $^1O_2$ and $OH^{\cdot}$, which are very toxic. When H_2O_2 accepts an unpaired electron from a reduced transition metal like iron (Fe^{2+}) or

copper (Cu^{2+}) it produces $OH^{\cdot}$ and HO by a Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + HO$). Therefore the toxicity of O_2 and H_2O_2 will depend on the availability and distribution of these transition metals[361, 362].

4.1.2.1 ROS production

ROS are produced by various enzymatic systems, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, the nitrogen oxides, xanthine oxidase and peroxisomes [363].

Approximately 1 or 2% of the O_2 molecules consumed in electron transport generate species such as $O_2^{\cdot-}$ and H_2O_2 [364, 365] mainly through side reactions catalyzed by the mitochondrial respiratory complexes I [366] and III [367-369].

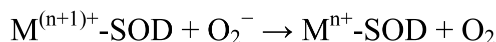
ROS production is balanced by the antioxidant molecules. This balance is essential considering that an unbalance will produce oxidative stress [370] damaging DNA, proteins and lipids. Oxidative stress may cause a dysfunction of Na^+/K^+ and Ca^{2+}-ATPases, alteration of glucose and Glut transport, excessive Ca^{2+} influx, ATP depletion, membrane depolarization and translocation of cytochrome c to the cytoplasm [371]. These deleterious effects can be reversible if they are repaired or by exchanging the damaged molecules. However, when the ROS are overproduced or the antioxidant systems are overcome, cells can die [372].

4.1.3. Antioxidant defences

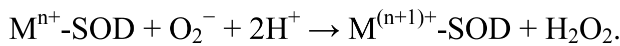
The most important protective systems against the ROS effect are the SOD, catalase and glutathione (GSH) enzymes [373, 374].

4.1.3.1 SOD

SOD is a mitochondrial enzyme that catalyzes the conversion of $O_2^{\cdot -}$ to H_2O_2. There are three major families of SOD, depending on the metal cofactor: Cu/Zn (which bind both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type, which binds nickel.



$$M^{(n+1)+}\text{-SOD} + O_2^{-} \rightarrow M^{n+}\text{-SOD} + O_2$$



$$M^{n+}\text{-SOD} + O_2^{-} + 2H^{+} \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2.$$

Reactions catalyzed by SOD

M = Cu *(n=1);* Mn *(n=2);* Fe *(n=2);* Ni *(n=2). In this reaction the* oxidation state *of the metal cation oscillates between n and n+1.*

4.1.3.2 GSH

GSH peroxidase and GSH reductase works together. GSH peroxidase is a cytosolic enzyme that degrades H_2O_2 generating two reduced GSH. These two GSH, form a disulphide bridge between them producing the GSSG and releasing two H^+ atoms that will eventually form a water molecule. In the presence of NADPH, GSH are quickly formed by the GSH reductase.

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

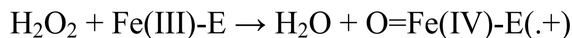
Reactions catalyzed by GSH peroxidase

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH$$

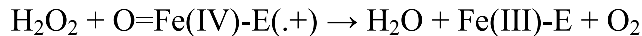
Reactions catalyzed by GSH reductase

4.1.3.3 Catalase

The catalase, an enzyme located in peroxisomes, destroys the H_2O_2 by dismutation.



$$H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E(.+)$$



$$H_2O_2 + O{=}Fe(IV)\text{-}E(.+) \rightarrow H_2O + Fe(III)\text{-}E + O_2$$

Reactions catalyzed by SOD

Fe()-E represents the iron *center of the* heme *group attached to the enzyme. Fe(IV)-E(.+) is a mesomeric form of Fe(V)-E, meaning that iron is not completely oxidized to +V but receives some "supporting electron" from the heme ligand. This heme has to be drawn then as radical cation (.+).*

4.1.3.4. Thioredoxins

Thioredoxins are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. This small redox protein is kept in the reduced state by the flavoenzyme thioredoxin reductase, in a NADPH-dependent

reaction; and acts as an electron donor to peroxidases and ribonucleotide reductases [375, 376].

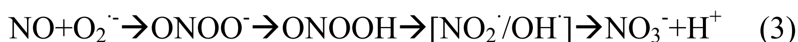
There are also other physiological systems that scavenge free radicals like uric acid, bilirrubine or albumin. Moreover vitamins C and E, flavonoids and other compounds derived from plants are powerful antioxidants .

4.2. Nitrative stress

NO undergoes different reactions in biological fluids resulting in the formation of NO_2^-, NO_3^- and $ONOO^-$.

$$2NO + O_2 \rightarrow N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (1)$$

$$NO + NO_2^- \rightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (2)$$



$$NO + O_2^{\cdot -} \rightarrow ONOO^- \rightarrow ONOOH \rightarrow [NO_2^{\cdot}/OH^{\cdot}] \rightarrow NO_3^- + H^+ \quad (3)$$

During the formation of NO_3^- (3) highly reactive intermediate products such as $NO_2^{\cdot}$ and $OH^{\cdot}$ are generated [377]. NO also reacts with $O_2^{\cdot -}$ to produce $ONOO^-$ [378], which mediates the major NO neurotoxic effects [379]. The affinity of $O_2^{\cdot -}$ is higher for NO than for SOD [380-382] avoiding the action of antioxidant systems; therefore the amount of NO and its diffusion coefficient are the limiting factors in the production of $ONOO^-$. Under physiological conditions $ONOO^-$ has a half life of 1-2s and an action radius of 100 µm, being sufficiently stable to diffuse through a cell to react with a target. It is degraded into multiple toxic products [383] or scavenged by the reaction with bicarbonate to produce nitrosoperoxycarbonate ($ONOOCO_2^-$) [384]. $ONOO^-$ is a powerful oxidant and is particularly

efficient at oxidizing iron-sulfur clusters, zinc-fingers, and protein thiols. This reaction contributes to cellular energy depletion. Moreover, $ONOO^-$ is one of the main protein-nitrating molecules [385, 386]. This nitration consists in the addition of a NO_2 to proteins, mainly with Tyr residues generating a 3-nitrotyrosine (**Fig.9**) The local environment of the Tyr is important in order to be nitrated, since the proximity of negatively charged residues increases the susceptibility of nitration [387]. This though, is not a massive process since the nitration under inflammatory conditions affects 1 to 5 of every 10,000 Tyr [388].

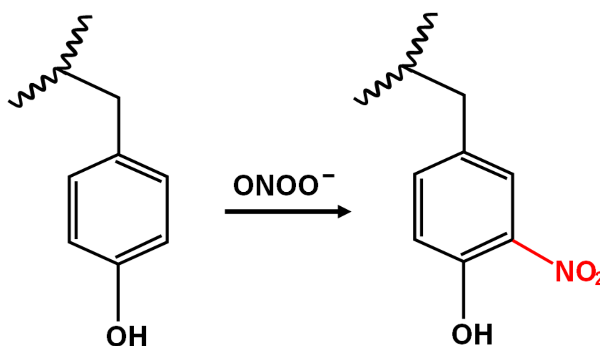


Figure 9. ***Nitrotyrosination process***

$ONOO^-$ mediated nitration depends on its secondary products ($NO_2^{\cdot}$) when is protonated to the acidic ONOOH (3 and 4) [389]:

$$ONOO^- + H^+ \rightarrow ONOOH + NO \rightarrow NO_2^{\cdot} + NO_2 \quad (4)$$

Nitrotyrosination of proteins alter its normal activity as was already discussed in the section 2.1. In the presence of high amounts of $ONOO^-$ apoptosis occurs but this process is not observed at

physiological concentrations. This is at least applicable in endothelial and mononuclear cells [390]. ONOOH and its intermediate products ($NO_2^{\cdot}$ and $OH^{\cdot}$) act also as oxidant agents. $NO_2^{\cdot}$ oxidizes certain amino acids such as Lys, His, Cys or Met.

4.3 Glycative stress

Glycation, also called non-enzymatic glycosylation, is a process that contributes to the post-translational modification of proteins [391]. This chemical reaction is a covalent interaction between an amino group and a carbonyl group of reducing sugar. Glycation occurs in the N-terminus of all amino acids, not only on the side chains of Lys and Arg, but also on Cys and His. As a result of glycation, advanced glycation end-products (AGE) are produced. AGE formation in proteins is irreversible and causes protease resistant cross-linking of peptides and proteins, often leading to protein deposition and amyloidosis [392, 393]. The carbonyl stress results from an impaired balance between the generation of carbonyl intermediates and the efficiency of the scavenger pathway.

4.3.1 Glycative agents

Among physiologically relevant sugars involved in glycation, glucose is the less reactive. The order of reactivity for the other monosacharides increases from hexoses to trioses and dicarbonyl compounds by several orders of magnitude. Only glucose in its aliphatic form (accounting only for the 0.001% of total glucose concentration) is involved in glycation reaction whereas α-oxoaldehydes are characterized by extremely high chemical activity

(up to 20.000-fold more reactive than glucose). The α-oxoaldehydes are intermediary metabolites of α-β-dicarbonyl structure, being endogenous potent glycating agents that produce the carbonyl stress.

4.3.1.1 Dicarbonyls and α-oxoaldehydes

Dicarbonyls are formed as glycolytic intermediates in the metabolic conversion of glucose, via a Maillard reaction by the degradation of glycated proteins [394] and lipid peroxidation. In a Maillard reaction (**Fig.10**), the first formed Schiff base adduct is converted to a more stable Amadori rearrangement product. In a further cascade of chemical reactions these Amadori products undergo several rearrangements to form irreversibly bound AGEs. The intermediate stage of the Maillard reaction is characterized by the formation of numerous secondary products. The sugar moiety of an early glycation product can undergo different chemical reactions, consequently producing low molecular weight carbonyls, α-oxoaldehydes, such as methylglyoxal (MG), glyoxal and 3-deoxyglucosone.

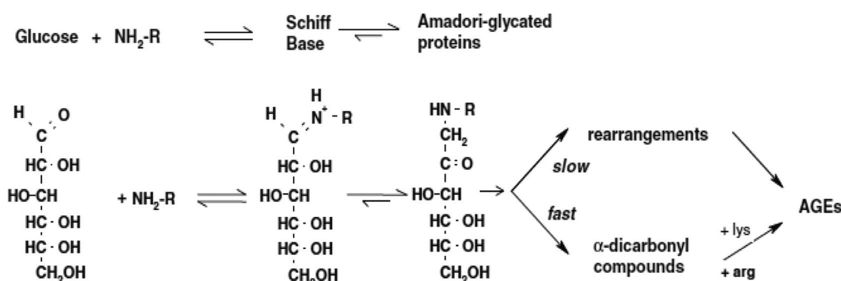


Figure 10. ***Maillard reaction***

The α-oxoaldehydes are generated intracellularly through both enzymatic and non-enzymatc pathways. Then they cross the cellular membrane, probably by passive diffusion. The concentrations of α-oxoaldehydes in human tissues and body fluids are usually low. Glyoxal and MG in human blood samples were 211 and 80 pmol/g respectively [395]. Increases production of MG arises mainly from hyperglycemia [396] when cells accumulates high level of glucose. This might be additionally exacerbated or by low glyceraldehyde-3-phosphate dehydrogenase activity [397] or as a consequence of decreased clearance by detoxification pathways. Several enzymes are involved in the detoxification of MG and make a network of four recognized catabolism pathways: the glyoxalase system, aldose reductase, betaine aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase [398].

4.3.2 Effects of AGE-modifications

Through their effects on the functional properties of extracellular matrix, intracellular signal transduction and protein function, AGEs may contribute to the pathogenesis of diabetic retinopathy [399],

neuropathy [400], renal failure [401-403] and macrovascular disease [404]. The effects of α-oxoaldehydes can modify irreversibly also DNA and RNA and these derivative nucleic acids may promote cell apoptosis as they are associated with mutagenesis.

A mechanism by which AGE-modified proteins may exert their effect is by binding to RAGE [405]. This has been identified on a variety of cells including endothelial and smooth muscle cells. Also AGE-modified proteins can be internalizated and degradated via monocyte/macrophage RAGE. In fact, increased RAGE expression has been found on endothelial cells, vascular smooth muscle cells and cardiac myocytes of diabetic patients[406]. It has been reported that ligation of AGE and RAGE causes activation of intracellular signalling, gene expression, and production of proinflammatory cytokines and free radicals, thus playing an important role in the development and progression of diabetic micro- and macroangiopathy [407].

Inside the cells, the impact of glycation is countered by the high turnover and short half-life of many cellular proteins. Long-lived extracellular proteins, however accumulate glycation adducts with age [408, 409]. Some of these adducts may be removed by enzymatic repair mechanisms, whilst all are removed by degradation of the glycated protein. Degradation of extracellular glycated proteins also requires specific recognition and internalization by RAGE [410] and posterior proteolytic processing.

4.4. Nitro-oxidative stress in brain

The brain has the highest oxygen metabolic rate of any organ in the body, consuming approximately 20% of the total amount of available oxygen [411]. This enhanced metabolic rate leads to an increased probability of ROS production and makes the brain more vulnerable to oxidative stress [412]. Brain also shows high concentrations of polyunsaturated fatty acids, which can be peroxidated. It also presents many enzymatic activities related with transition metals, which can catalyze the free radical production[413].

4.5 Nitro-oxidative stress in AD

The free radical toxicity is evident in AD patients, who show dramatic evidences of oxidative, glycative and nitrative stress in hippocampus, cortex [414-417], and in CSF [418]. In the context of AD, the Aβ fibrils induce ROS formation [137, 419]. Similarly, chronic $A\beta_{1-40}$ intracerebroventricular infusion causes $ONOO^-$ formation [420], which correlates with the increased nitrotyrosination observed in AD. This is explained by the fact that Aβ stimulates the reactive microglia surrounding the senile plaques to produce $ONOO^-$ through the induction of iNOS expression [421-423]. Actually, iNOS and endothelial NOS are more expressed in astrocytes associated with neuritic plaques [424-426] while nNOS is strongly related with NFT and plaques [427]. Indeed, some of the deleterious outcomes observed in AD patients may be explained by the effects of nitrotyrosination on glucose metabolism, cytoskeletal integrity, antioxidant defense or protein turnover [428, 429].

The effect of ROS in the limitation of glucose flux provokes high concentrations of MG in AD [430-432]. To worsen this situation MG detoxification by glyoxilase is decreased because GSH is needed as a cofactor, and there is increasing evidence supporting that GSH is depleted in AD [433, 434]. Likewise, AGE with unchelated transition metals contribute to AD pathology inducing cross-linking and fostering the aggregation of Aβ [435]. For this reason and for the impairment of lysosomal proteases activity in AGE modified proteins [436, 437], AGE could aggravate the inability of phagocytes like microglia to clear plaques. This increase in AGE might contribute to the inflammatory activation of the microglia. It can also mediate cytotoxicity by free radical formation [438, 439] or through ATP depletion [440-442]. AGE is a biomarker of AD and CAA, being present in NFT [443], in early stages of amyloid plaques [444] and in CSF [445].

5. Albumin

Human serum albumin (HSA) is the most abundant plasma protein[446] being about 60% of the total plasmatic proteins [447]. It is also the major protein of CSF, which is in part an ultrafiltrate of plasmatic proteins [448].

5.1. Biochemistry of albumin

HSA is a single peptide chain of 585 amino acids, without prosthetic or carbohydrate group and characterized by the presence of 35 Cys residues which forms seventeen disulphide bridges with the exception of Cys 34 that has a free sulphydryl group [449]. The

disulphide bridges confer the necessary stability to the protein in the blood to avoid dissolution. Moreover, albumin contains a high amount of ionic amino acids, especially Glut (20 residues) and Lys (60 residues), confering a relatively high solubility to the protein. HSA has a secondary structure with 67% of α-helix, 23% of extended chain and 10% of β-sheet [446, 450, 451]. Albumin is a flexible molecule, with the ability of changing its shape depending on the environmental conditions such are temperature, pH or ionic strength [452-454].

HSA consists of a monomeric globular protein with three domains I, II and III [446, 455, 456]. The wide range of compounds that bind albumin can be accommodated in the domains II and III because both have a pocket formed mostly of hydrophobic and positively charged residues. This is the explanation of the higher affinity of albumin for hydrophobic anionic compounds [453, 457].

5.2. Physiological properties

HSA is synthesized by the liver and circulates in blood (3.5-5g/dL) during 21 days [458] until it is degraded by the liver and kidneys [459, 460]. In brain microglia is able to produce albumin, explaining the high proportion of albumin in CSF [461].

Albumin is working in the maintenance of oncotic or colloid osmotic pressure [462, 463] and in the transport of different molecules such are hormones [453], free fatty acids [464, 465], bilirrubin [466], NO[467], Ca^{2+} and other ions [468-474], or some drugs as penicillines[453], warfarin[475] and ibuprofen [476].

A neuroprotective role is also among the functions described for HSA. It has been involved in neuronal survival[477] by increasing the synthesis and release of Glut [478], also in the prevention of neuronal death[479-481] and apoptosis through a reduction of excessive cytosolic Ca^{2+} concentrations [482]. It can also act by scavenging oxidative stress. To perform its antioxidant properties, HSA quenches metal ions, ROS and NO [453, 467] through its free Cys [483-487]. Accordingly, the homolog protein in bovine, bovine serum albumin (BSA), exhibits SOD-like activity by inhibiting $O_2^{\cdot -}$ formation, reducing lipid peroxidation [488, 489], leading to a decrease in the cellular oxidative stress. Together with its antioxidant properties, BSA exhibits an antimutagenic effect against certain genotoxic compounds [490]. Otherwise, HSA has an N-terminal region, the copper-chelating tetrapeptide aspartate-alanine-histidine-lysine (DAHK), that attenuates DNA strand breaks and telomere shortening [491].

As well, in stroke HSA has a positive effect: it decreases edema and infarct size [492], increases local perfusion and maintains microvascular integrity [493].

5.3. Albumin and Aβ

Albumin binds almost all of the $A\beta_{1-40}$ and $A\beta_{1-42}$ present in the circulation [494-497]. This places HSA as a relevant player in AD, since it is proposed that by binding to Aβ it inhibits its aggregation[498-502]. The equilibrium between the cerebral and plasmatic Aβ is critical for AD onset and it is controlled by two receptors. The LRP releases Aβ from the brain to the blood [503, 504] and the RAGE moves

the systemic Aβ to the brain [505-507]. Interestingly, the injection of antibodies in the CNS produces an increased Aβ efflux to the plasma as a result of the resolubilization of the Aβ aggregates [508, 509]. This finding could have important implications for Aβ-albumin interaction and Aβ clearance [461, 510].

5.4. Pathologies related with albumin dysfunction

The main diseases related with albumin are due to changes in its concentration. Hypoalbuminemia is a reduction of albumin concentration (1-2.5 g/dL) that can be caused by several pathologic situations such are: a) liver disease, being cirrhosis the most common [511, 512], b) an excess of excretion by kidneys, as occurs in nephrotic syndrome [513], c) an excess of loss by bowel, in a protein-losing enteropathy like Ménétrier's disease [514, 515], d) by its loss in the absence of the skin barrier in burns patients [516]), e) hemodilution in pregnancy [517], f) increased vascular permeability [518], g) decreased lymphatic clearance, h) acute diseases states as negative acute-phase protein [519] or i) rare mutation causing analbuminemia [520, 521]. On the other hand, the increase in albumin concentration, or hyperalbuminemia, is a sign of severe or chronic dehydration [522] or high protein diets [523].

Different physiological or pathological factors like age, diseases, genetic aspects or the binding to endogenous ligands and xenobiotics, change HSA binding site properties contributing to its intra- and interindividual variability [524].

In normal conditions HSA suffers nitro-oxidative modifications, such as glycation [525], but these are enhanced by aging. Albumin has

many Lys and Arg (23 residues) that make it an excellent target for glycation [526] and the Tyr (18) that makes it susceptible for nitrotyrosination [527]. Due to its relevance in diabetes mellitus[528], HSA glycation has been widely studied demonstrating dramatic changes in its structure and function [529-531]. In fact it is known that glycation induces refolding of albumin into β-sheet [532] being toxic for neurons, leukocytes, pericytes and endothelial cells[533-538].

II. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

NO is an important molecule playing a key role in the physiology of memory processes in the brain as well as in the regulation of vascular tone. During memory consolidation, its function contributes to the synaptic strengthening produced in LTP. How NO participates in the LTP process is explained by its activity as a retrograde messenger from postsynaptic to presynaptic endings, facilitating the bidirectional communication between neurons and enhancing its interaction during synapses. Regarding this situation, we propose NO as a candidate to regulate the expression of one of the key proteins in synaptic events: the NMDARc.

In pathological situations, under a pro-oxidant environment like that happening in AD, NO can interact with $O_2^{\cdot -}$, forming the $ONOO^-$, which modifies proteins. All together contributes to the oxidative, nitrative and glycative stress in AD. The alteration of proteins under this stressful condition can tell us about the state of the disease progression at both sides of the BBB: brain parenchyma and blood.

2.2 Objectives

The work embraced in this thesis addresses the issue of the role of NO in synapses and in nitro-oxidative stress in AD. The main goals are the following ones:

2.2.1 The study of the NO effect in NMDARc expression in neurons: NMDARc are the most relevant receptors in LTP in glutamatergic neurons. The differential expression of its subunits can affect Ca^{2+} entry triggering survival or pro-apoptotic pathways.

GluN2B translation has to be tightly controlled due to its dual role in synapses and extrasynapses. We attempt to link the effect of NO in the GluN2B translational activation through the neuronal eIF2α phosphorylation.

2.2.2 The study of the effects of oxidative, nitrative and glycative stress in albumin: Albumin is the main protein in blood circulation and CSF. Modification by nitrotyrosination and glycation can impair its physiological function converting it in cytotoxic. These posttranslational modifications may turn albumin to an ineffective or even to a harmful protein. Herein we deal with the study of the effects of albumin nitration and glycation in AD.

III. RESULTS AND METHODS

CHAPTER I:

Nitric Oxide Induces the Expression of the Glutamatergic GluN2B subunit by Reverting the mRNA 5’UTR Translational Repression

Nitric Oxide Induces the Expression of the Glutamatergic GluN2B Subunit by Reverting the mRNA 5'UTR Translational Repression

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Running Title: Nitric oxide induces GluN2B translation

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Abstract

Glutamate (Glut), the main neurotransmitter of the excitatory synapses, activates the N-methyl D-aspartate Receptor (NMDARc) producing long term-potentiation (LTP) in cortex and hippocampus to consolidate memory and learning process.
This channel is formed by a heterotretamer composed mainly by two GluN1 and two GluN2 subunits. The subunit GluN2B is playing a key role in NMDARc synaptic function and its dysregulation is associated to cognitive impairment and neurodegenerative diseases. GluN2B mRNAs has a 5'untranslated region (5'UTR) with three upstream AUG (uAUG) which act as repressor of its translation. Under determined stimulus, the de-repression occurs through the eukaryotic initiation factor 2 α (eIF2α) phosphorylation mediated by several kinases. Here we demonstrate by immunofluorescence (IF) and westernblotting (WB) that Glut produces an increase of GluN2B expression. Nitric oxide (NO), as well as a NMDA agonist and an eIF2α phosphatases inhibitor, produces an increase in the GluN2B expression and eIF2α phosphorylation. Consistently luciferase experiments show that these treatments prevented the UTR repression of GluN2B. We identified the heme regulated eIF2α kinase (HRI) as the kinase activated by NO that phosphorylates eIF2α, and this effect as well as the GluN2B expression was blocked by a HRI inhibitor used in cortical mouse neurons and in mice. The function of this receptor was assayed by electrophysiological techniques in cortical mouse neurons and synaptosomes showing a high Ca^{2+} entry when treated

with NO. In conclusion, our data suggest that Glut activates GluN2B translation, through the NO production. It activates HRI, which phosphorylates eIF2α reverting the 5'UTR GluN2B repression.

Nonstandard abbreviations used: 5'UTR: 5'untraslated region; 7-NI: 7-nitroindazole; Abs: antibodies, AUC: area under the curve; BIC: bicuculline; BSA: bovine serum albumin; Ca^{2+}: calcium; CHX: cycloheximide; eIF2α: eukaryotic initiation factor 2 α; Glut: glutamate; HRI: heme regulated eIF2α kinase; IF: immunofluorescence, iHRI: inhibitor of HRI; ISO: isotonic solution; LTP: long term potentiation; NMDARc: N-Methyl D-Aspartate Receptor; nNOS: neuronal NOS; NO: nitric oxide; NOS: NO synthase; PSD-95: postsynaptic density -95; Sal: salubrinal; Ser: serine; SAP-102: synapse associated protein 102; SN: supernatant; SNP: NO donor; uAUG: upstream AUG; uORF: upstream open reading frame; WB: western blot; WT: wild type.

Introduction

N-Methyl D-Aspartate receptor (NMDARc) is an excitatory receptor activated by ligand and voltage (Engberg et al., 1978; MacDonald and Wojtowicz, 1982; Mayer et al., 1984; Nowak et al., 1984) that allows mainly the calcium (Ca^{2+}) entry in neurons (Dingledine, 1983; Mayer and Westbrook, 1987). This glutamatergic neurotransmission plays a key role in synaptic plasticity and memory due to its implication in long term potentiation (LTP) in cortex and hippocampus (Collingridge et al., 1983; Harris et al., 1984). It produces the activation of intracellular pathways with short-term effects, such as the activation of calmodulin and nitric oxide (NO) production and long-term effects like the gene expression through of cAMP response binding-element phosphorylation (Ghosh et al., 1994).

NMDARc has three subunits: GluN1, GluN2 and GluN3 (Moriyoshi et al., 1991; Constantine-Paton and Cline, 1998; Das et al., 1998). The functional receptor is composed of multiple GluN1 subunits in combination of at least one type of GluN2 (Monyer et al., 1994). The subunit GluN2B is highly expressed in the early stages of development when synapses are growing (Loftis and Janowsky, 2003) interacting directly with the postsynaptic density - 95 (PSD-95) scaffold protein and through PSD-95 with other key proteins in glutamatergic transmission such as neuronal NO synthase (nNOS) (Brenman et al., 1996).

The expression of GluN2B in the synapses is regulated at the translational level. GluN2B mRNA has a long 5'untraslated region

(UTR) with 179 pb, containing three upstream AUG (uAUG). Under normal conditions the translation of mRNAs with long 5'UTR, GC rich content, extensive secondary structure, uAUG and more than one upstream open reading frame is repressed (Kozak, 1987). This regulatory system was described for GCN4 in yeast (Mueller and Hinnebusch, 1986; Qiu et al., 2000) and it is shared with different 5'UTR transcripts that are translationally activated by particular situations like cellular stress. During the initiation of the translation, the small subunit of the ribosome with the ternary complex formed by tRNA coupled to methionine, eukaryotic initiation factor 2 (eIF2) and GTP scans the 5'UTR of mRNA to detect the main AUG. Under particular situations directed to avoid the general protein translation, eIF2α subunit is phosphorylated in serine (Ser) 51 binding eIF2B. Then eIF2B is mostly bound to p-eIF2α and it cannot exchange the GDP for GTP and it reduces the inhibition of the translation because just the ternary complex coupled to GTP is active (de Haro et al., 1996). But in proteins with 5'UTR that contains more than one uAUG, the phosporylation of eIF2α produces a less availability of active ternary complex making more efficient the scanning process, reaching more easily the main open reading frame, thus derepressing the 5'UTR translational inhibition.

There are four main kinases involved in the eIF2α phosphorylation but we focus our research in heme regulated eIF2α kinase (HRI). HRI is activated by NO due to its interaction with the heme group (Yun et al., 2005). In this work, we have studied the role of the NO

in NR2B translational derepression through HRI activation and eIF2α phosphorylation in glutamatergic signalling.

Matherials and methods

Mouse embryo cortical cell cultures

Cortical cells were isolated from 18 day-old CB1 mouse embryos. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mediques-Universitat Pompeu Fabra. Hippocampus and cortex were aseptically dissected and trypsinized. Cells were seeded in DMEM (Gibco, USA) plus 10% horse serum (Gibco) into 1% poly-L-Lysine (Sigma-Aldrich, USA) coated coverslips ($5x10^4$cells/cover). After 2 h, medium was removed and neurobasal medium was added containing 1% B27 supplement (Gibco) plus antibiotics. Cultured hippocampal cells were used for the experiments on day 7 for the Ca^{2+} experiments and 10 for WB and IF (Kaech and Banker 2006).

Human brain samples

Human brain tissue samples were supplied by the Banc de Teixits Neurològics (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona), the Unitat d'Anatomia Patològica (Hospital del Mar) and the Unitat de Neuropatología y Banco de Cerebros (Fundación Hospital Alcorcón). The procedure was approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Brain sample was obtained from the frontal cortex of a healthy aged

individual. Brain sample was lysated with a cocktail containing NP40 lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 0.05% aprotinin, 1mM dithioltreitol) and a 1x of protease inhibitors (Complete mini-EDTA free, Roche Diagnostics GmbH, Switzerland). The mix was mechanically disaggregated with plastic micropistilles in a 1 mL eppendorf and 1mL syringe. The brain solution was centrifugued at 17,500 x g for 10 min. The supernatant (SN) was quantified by the Bicinchoninic Acid (BCA; Pierce® BCA Protein assay kit, Thermo Scientific, USA) assay.

Mice treatments

The inhibitor of HRI (iHRI; Janssen Research & Development, Belgium) used at 50 mg/kg, the inhibitor of nNOS, 7-nitroindazole (7-NI) (Sigma-Aldrich), used at 50 mg/kg, and dimethyl sulfoxide (DMSO; Sigma-Aldrich), as vehicle control were administered after the training session intraperitoneally (i.p.) in a volume of 2 mL/Kg. Synaptosomes were isolated from mouse hippocampus.

Preparation of cortical and hippocampal synaptosomes

Cortical and hippocampal synaptosomes were obtained as described by Myhre and Fonnum, 2001 with minor modifications. Two mice were decapitated, their brains were rapidly removed, and the cortex and hippocampus were dissected and homogenized in cold homogenization buffer (5 mM Tris–HCl and 320 mM sucrose; Sigma-Aldrich), using a borosilicate glass homogenizing tube fitted with a Teflon pestle and filtered through two layers of surgical gauze. The homogenate was centrifuged at 1,000 × *g* at 4 °C for 10

min. The SN was recovered, and sucrose buffer was added to a final sucrose concentration of 0.8 M. Samples were then centrifuged at 13,000 x g for 30 min at 4 Cº. The SN was discarded and the synaptosomes layer was separated from mitochondria by carefully adding 1 mL of ice-cold 320 mM sucrose buffer and gently shaking. Finally, the synaptosomes fraction was diluted in Hank's balanced saline solution (HBSS) buffer (Gibco) to a final protein concentration of about 0.1 mg/mL. Protein concentration was determined using Bio-Rad protein reagent (Bio-Rad, USA). The final synaptosomes suspension was distributed in 1 mL aliquots to perform the experiments. Synaptosomes integrity was assessed by electron microscopy (Fig. 1B).

Cloning of GluN2B 5'-untranslated region (UTR)

Total RNA was extracted from hippocampus of brain tissue, and one-step RT-PCR was carried out using kit (Qiagen, Germany) with primers designed to amplify GluN2B 5'UTR: 5'-CATTTATCCTTCGTCTTTCTTATGTG-3' , 5'-CAACACCAACCAGAACTTG- 3'. PCR product, a single band matching the molecular weight of GluN2B 5'UTR (~180 nt), was isolated and purified from an agarose gel using the Ilustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Helthcare, UK) and stored at -20ºC for further uses. The 5'UTR DNA fragment was then inserted into the HindIII site of a modified pGL4.10 [luc2] vector (Promega, USA) containing the CMV promoter cloned at BglII and HindIII sites.

Generation of GluN2B 5'UTR mutants

5'UTR mutations were generated by site-directed mutagenesis (QuikChange® II XL site-directed mutagenesis kit, Stratagene). The A from the first and second ATG were substituted for a T with the primers: 5'-CCTTCGTCTTTCTTTTGTGGATTTGCAAGCGAGAAGAAGG G-3' and 5'-CCCTTCTTCTCGCTTGCAAATCCACAAAAGAAAGACGAAG G-3'. This construct was used like a template for exchange the A from the third ATG for a T with the following primers: 5'-CTGGACATTCCCAACTTGCTCACTCCCTTAATCTG-3' and 5'-CAGATTAAGGGAGTGAGCAAGTTGGGAATGTCCAG-3'. The incorporation of the mutations was verified by sequentation.

Transient DNA transfection of SH-SY5Y cells and luciferase assay

SH-SY5Y neuroblastoma cells were seeded in 96-well plates at a density of 15,000 cells per well and grown for 12 h with DMEM plus 10% fetal bovine serum (Gibco). Afterwards, a total of 250 ng of DNA was transfected into each well, adjusting to the following conditions: 250 ng of pcDNA3 plasmid as blanks, 25 ng of renilla plus 25 ng of CMV-luciferase Vector plus 200 ng of pcDNA3 as controls, and finally 25 ng of renilla + 25 ng of GluN2B-5'UTR CMV-luciferase construct plus 200 ng of pcDNA3 as problem samples. Cells were transfected using JetPEI transfection reagent (PolyPlus, Korea) for 4h. Afterwards, medium was changed and cells were incubated for 24 h to allow sufficient gene expression. After 1h treatments with Sodium Nitroprusside (SNP; Sigma-

Aldrich), NMDA plus glycine (Gly) (Tocris, UK) and salubrinal (Sal; Calbiochem, USA) cells were lysed and luciferase and renilla activities were measured by using the Dual-Glo™ Luciferase Assay System (Promega) following manufacturer's instructions. The luminescence was read using the plate luminescence reader (Fluostar OPTIMA, BMG labtech, Germany).

Cellular and synaptosome treatment

SNP (Sigma), NMDA plus Gly, -(-)Bicuculine methiodide (BIC; Tocris), BIC plus 4-Aminopyrimidine (4-AP; Sigma) were diluted in neurobasal medium and incubated at indicated concentration (100 nM SNP, 100 µM NMDA plus 100 µM Glycine, 50 µM BIC 2.5 mM 4-AP) with cortical neurons and synaptosomes (diluted in HBSS instead neurobasal medium) for 1h at 37ºC. Sal and dybutyril GMP cyclic (dbGMPc; Sigma-Aldrich) were dissolved in DMSO, and then it was diluted in neurobasal medium to treat cells at 100 µM for 1h at 37ºC. L-Glutamic acid (Glut; Sigma-Aldrich) was dissolved in HCl 1 M and then diluted in HBSS at 10 µM to treat synaptosomes and mouse cortical neurons for 1h. The 7-NI, cycloheximide (CHX), iHRI, BAPTA·AM and threo ifenprodil hemitartrate (If; Tocris) were diluted in DMSO and these stock solutions were rediluted in neurobasal medium. The NMDA blocker, MK-801 (Tocris), was diluted in neurobasal medium and incubated in cells at 10 µM. Cortical cells with 10 µM 7-NI, 10 µM MK-801, 10 µM If, 10 µM BAPTA·AM and 1µM iHRI were pre-incubated for 30 min and then the inhibitors were incubated plus the

stimulators SNP, NMDA plus Gly, Glut or BIC plus 4-AP for 1h at 37ºC.

RT-PCR and PCR to amplify the HRI through mRNA levels

RNA extraction (Nucleospin RNA II kit, Macherey-Nagel) from human cortex and mouse cortical neuron was carried out and RT–PCR was performed using SuperScrip-RT system (Invitrogen). Aliquots of 1 µg cDNA were used as template for PCR. The primers used to amplify HRI from human were: 5’-CCCCGAATATGACGAATCTG-3’ and 5’-CAGATTCGTCATATTCGGGC-3’; the primers used to amplify HRI from mouse were: 5’-GAAGTGGGTTTGGTTCATGC-3’ and 5’-GCATGAACCAAACCCACTTC-3’. PCR conditions for all transcripts were: 95°C for 3 min; 95°C for 30 s; 60° for 30 s, 72°C for 30 s; 72°C for 7 min; with 35 cycles of amplification. The amplified HRI was resolved in a 2% agarose gel.

Protein levels detection by western blot (WB)

After treatment, cortical cells were washed twice with PBS and they were detached mechanically with a scrapper and lysed with NP40 buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 0.05% aprotinin, 1 mM dithioltreitol) and a 1x of protease inhibitors (Complete mini-EDTA free from Roche Diagnostics GmbH). The lysate was centrifuged at 17,500 x g for 10 min and the protein concentration was measured by BCA assay. Protein (100 µg) was mixed with loading buffer 5x and boiled 5 min at 100ºC. Samples

were resolved in 8% polyacrilamide gels. Gels were transferred in polyvinylidene fluoride membranes (ImmobilonP, Millipore, USA). Blocking solution, tween-tris buffer saline (TTBS)-5% milk, was added in membrane for 1h. Membranes were incubated overnight (o.n.) at 4ºC with the primary antibodies (Abs). Primary Abs were incubated with the following dilutions: mouse GluN2B Ab (Neuromab, USA) 1:5 in TTBS; rabbit phospho-eIF2α (p-eIF2α Ab) (Abcam, UK) 1:500 in TTBS-5% bovine serum albumin (BSA); mouse eIF2α total Ab (Abcam) 1:500 in TTBS-5% milk; mouse actin Ab (Sigma-Aldrich) 1:4000 in TTBS-5% milk. Membranes were washed thrice with TTBS to release the excess of antibody. Anti- mouse and anti-rabbit secondary Abs (GE-Healthcare) at 1:4000 dilutions with TTBS-5% milk were respectively added in membranes and stirred for 1h. Three washes with TTBS were performed. Membranes were revealed with Supersingal West Pico and Femto Chemiluminiscent substrate (Thermo Scientific Pierce). Blotting quantification was done with Quantity One software. GluN2B and p-eIF2α band intensity were normalized by actin and eIF2α total levels, respectively.

Immunofluorescence (IF) experiments

Cortical cells were seeded at 75,000 cells/well in poly-L-lysine (Sigma-Aldrich) coated coverslips. Coverslips were washed twice with PBS Ca^{2+} Mg^{2+} after being treated. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, and three washes with PBS Ca^{2+} Mg^{2+} for 10 min each one, were done to remove PFA traces. Afterwards, cells were permeabilized with 0.1% Triton X-100 and

they were washed thrice with PBS Ca^{2+} Mg^{2+}. Coverslips were incubated with blocking solution (5% fetal bovine serum, 1% BSA and 0.02% sodium azide) o.n. at 4ºC. Subsequently, cells were incubated for 2 h at room temperature (RT) in a hydration chamber with 1:10 mouse anti-GluN2B Ab. After primary Ab incubation, cells were incubated with 1:1000 Alexa Fluor 488 goat anti-mouse Ab (Invitrogen) for 1 h at RT. Digital images were taken with a Leica TCS SP confocal microscope and analysed with Leica confocal software (Heidelberg, Germany).

Dendritic spine morphogenesis studied

Rat cortical neurons were treated with PBS (control) and 100 nM SNP in the absence/presence of iHRI for 1 h. Cells were fixed and incubated with anti-GluN2B Ab as previously explained and actin was detected by phaloidin in red. Images were obtained with a Zeiss Axiovert 200M LSM PASCAL Confocal Laser Scanning Microscope. High-resolution (2,048 × 2,048 pixel) fluorescence images were acquired with a LP530 emission filter and excitation at 488 nm.

Measurement of intracellular [Ca^{2+}] in cortical mouse neurons

Cytosolic Ca^{2+} signal was determined at RT in cells loaded with 4.5 μM FURA-2·AM (30 min) as previously described (Fernandes et al., 2008). Cytosolic Ca^{2+} increases are represented as the normalized ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation (FURA-2 ratio 340/380). All experiments were carried

out at RT and cells were bathed in an isotonic solution (ISO) containing (mM): 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 10 HEPES (300 mosmol/L, pH 7.4 with Tris). Cells were stimulated with BIC, NMDA plus Gly, and blocked with If plus BIC and 4-AP, as it showed in Fig 6.

HRI immunoprecipitation (IP) from human brains and cortical neurons

200 µg of homogenated brain and 200 µg of protein from cortical neurons were used for the IP. The samples were pre-incubated half an hour at 4ºC with protein G (GE Healthcare), which is previously washed with PBS. This step is needed to avoid unspecific protein binding with protein G. Then, the samples were centrifuged at 10,000 x g for 10 min. The SN was incubated o.n. with 5 µg of anti-HRI Ab (Abcam). Following the addition of protein G immobilized on sepharose (GE Healthcare), samples were shaken for 2 h at RT. HRI was pulled down by centrifugation at 14,000 x g for 10 min and washed thrice. 60 µl of loading buffer x5 was added to pellet and it was boiled for 6 min at 100ºC. HRI IP was recovered by centrifugation at 14,000 x g for 10 min. 30 µL of this sample was resolved in 8 % polyacrilamide gel. Gels were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). Membranes were blocked with TTBS-5% milk solution for 1h. HRI detection was performed incubating with HRI Ab diluted 1:1000 in TTBS-5% milk o.n. at 4ºC. Afterwards, three washes with TTBS for 5 minutes were done. 1:4000 rabbit anti-mouse Ab were used as secondary Abs at RT for 2 h to detect the primary Abs. Three

washes with TTBS for 5 min were performed and membranes were developed with the Supersignal West Femto Chemiluminiscent substrate.

Results

Glutamatergic signalling upregulates GluN2B subunit expression due to eIF2-α phosphorylation

Cortical neurons treated with Glut for 1h shown an increase of GluN2B expression as we obtained by IF and WB (Fig.1A and 1C), and this expression was prevented by BAPTA·AM, a Ca^{2+} chelator, suggesting that GluN2B expression was dependent on the Ca^{2+} downstream signalling. Since Ca^{2+} is activating nNOS, we treated mouse synaptosomes with Glut plus 7-NI, a nNOS inhibitor (Fig.1C and 1D). Consequently we obtained that nNOS inhibition prevented GluN2B expression suggesting that NO was directly involved. Then cortical neurons were treated with the NO donor, SNP, which yielded an increase in GluN2B expression (Fig. 1G and 1H). The increased GluN2B expression was produced in a SNP concentration dependent manner as we assayed in synaptosomes (Fig. 1E and 1F). The specific agonist NMDA also triggers the same effect (Fig. 1G and 1H) supporting that the observed effect was mediated by NMDARc activation. Interestingly the GluN2B mRNA has a 5'UTR with 179 bp and three ATG (acces number in NCBI: NM_000834) that would allow its translation just when eIF2α is phosphorylated. Then we studied the phosphorylation of this factor regarding GluN2B expression (Fig. 1E and 1G), demonstrating that

p-eIF2α levels are increased by SNP and NMDA treatment. Moreover similar results were obtained when cortical neurons were incubated with Sal (Fig. 1G, 1H), an inhibitor of the phosphatase PP1 that dephosphorylates p-eIF2α, raising the levels of p-eIF2α.
We asses the regulation of GluN2B mRNA translation by a luciferase reporter assay. We cloned GluN2B 5'UTR sequence under the control of a CMV promoter (Fig. 2A). GluN2B wild type (WT) 5'UTR produced a repression in the luciferase expression regarding to cells transfected with the vector that does not contain the GluN2B 5'UTR (Fig. 2B). To demonstrate the responsibility of the three uATG in the UTR luciferase repression, we developed a triple mutant (3x Mut) exchanging A for T. The triple mutant was able to recover the UTR repression significantly (Fig. 2B). Then cells transfected with the WT 5'UTR vector were treated with SNP and NMDA promoting a significant increase in luciferase expression (Fig. 2C). Treatment with SNP and Sal in cells transfected with the 3 x Mut 5'UTR vector produced a significant repression in luciferase expression (Fig. 2C) demonstrating that when the uATG are no present the p-eIF2α repress the general translation.

Role of HRI kinase in GluN2B increasing by NO

It is known that NO is able to activate HRI kinase in erythrocytes by the binding to the N-terminal heme binding domain (Yun et al., 2005). This interaction disrupts the inhibitory interactions between the heme binding domain and the catalytic domain, activating HRI that could phosphorylates eIF2α stimulating GluN2B expression.

HRI was firstly isolated in rabbit reticulocytes (Ranu and London, 1976), but is also present in other kind of cells. Here we probe that HRI mRNA and protein are expressed in mouse cortical primary neurons and human cortex (Fig. 3A and 3B).

To study the involvement of HRI kinase in the phosphorylation of eIF2α and the consequent GluN2B expression we treated mice with an inhibitor of HRI, iHRI (Fig. 3C, 3D). Mice treated with iHRI showed lower levels of GluN2B and p-eIF2α compared to controls treated with DMSO, the vehicle. The expression of GluN2B and the phosphorylation of eIF2α were also reduced when mice were treated with a nNOS inhibitor, 7-NI (Fig. 3C, 3D), avoiding the normal production of NO by neurons and its downstream effects. The iHRI also reduced the GluN2B increase and p-eIF2α in the cortical neurons treated with SNP or NMDA (Fig. 4A). Consistently with a translational regulation, the treatment with the translation inhibitor CHX impaired GluN2B expression when cells were incubated with SNP or NMDA (Fig. 4A). Moreover the dbGMPc, a GMPc analogue, was unable to produce a GluN2B increase (Fig. 4A), discarding that the obtained NO effects would be mediated by the classic activation of guanylate cyclase. The specific involvement of GluN2B in this glutamatergic signalling cascasde was assayed with If, a GluN2B blocker, which avoided the effect of NMDA in GluN2B expression produced by NMDA (Fig. 4A).

GluN2B expression by Glut and NO would have a main role in the maintenance of LTP. For this reason we also studied if this pathway was producing its effect in the synaptic spines (Fig. 4B). We found

that SNP induced an increased expression of GluN2B in rat cortical spines and this effect can be prevented by inhibiting HRI.

SNP treatment produces an increase in the Ca^{2+} entry

NO increases GluN2B translation but it is necessary to demonstrate that these receptors are functional (Fig. 5). We stimulate cells with 100 µM NMDA plus 100 µM Gly (Fig. 5A and B). Cells treated with SNP showed a higher Ca^{2+} entry when we compared the area under the curve (AUC) from the treated cells *vs* controls. This increase was avoided when we pre-incubated the cells with CHX (Fig.5A and B). Moreover the Ca^{2+} response was specific for GluN2B containing channels because the selective antagonist If was able to block it (Fig.5A and B).

To study the involvement of GluN2B increased translation by NO in synapses, we enhanced synaptic activity of cortical cells with a blocker of the inhibitory γ-aminobutyric acid receptors, BIC and 4-AP, a weak blocker of K^+ channels (Fig. 5 E and F). They stimulate synaptic activity by blocking inhibitory elements and depolarizing the cell, respectively. Cells treated with SNP, stimulated with BIC plus 4-AP shows more peaks each minute and a higher maximum peak that control ones (Fig. 5E, F, Table 1). This increase was mainly due to GluN2B subunit, since the application of If reduced the number of peaks each minute as the maximum peak in control and SNP treated neurons (Fig. 5E, F, Table 1).

GluN2B expression and p-eIF2α levels were higher when cortical cells were stimulated with BIC or BIC plus 4-AP for 1h (Fig. 5E, F). The MK-801, a blocker of NMDARc (Fig. 5C), and If, a

specific blocker of receptors containing GluN2B subunit, avoided the increase in GluN2B expression and p-eIF2α levels (Fig. 5D).

Discussion

NMDARc are important players in synapses and neuronal communication. It expression has to be tightly controlled since an excess in its activity can trigger neuronal excitotoxicity and neurodegeneration (Mody and MacDonald, 1995; Kemp and McKernan, 2002), and a lower activity can produce neuronal pathologies, which include cognition and memory deficits (Newcomer and Krystal, 2001; Rison, 1998). In addition, an abnormal low expression of NMDARc is related with schizophrenia (Duncan et al., 1999; Jentsch and Roth, 1999).

We focused in the study of GluN2B subunit because is involved in neuronal growth and would have a role in synaptic plasticity. GluN2B expression is highly regulated at the translational level (Gray and Wickens, 1998) since its mRNA has a long 5'UTRs containing three uAUGs that is highly conserved between species.

To consolidate memory and learning is needed the protein synthesis. Firstly there is an activation of the transcription of genes regulated by the cAMP response binding-element transcription factor to promote the growth of the dendrites and the synaptic spines (Ghosh et al., 1994). But transcription is a process that spends too much time, and in neuronal plasticity protein synthesis is needed quickly specially in LTP. For this reason, several mRNA are transported into dendrites waiting to be translated after synaptic

activity (Mayford et al., 1996; Bagni et al., 2000; Richter and Lorenz, 2002). In dendrites there are polyribosomes, ready to produce the translation of new proteins needed in short times. It has been reported that mRNA of GluN2A but overall GluN2B is located in neurites (Miyashiro et al., 1994; Quinlan et al., 1999). Then, the glutamatergic signalling will induce the rapid expression of GluN2B subunits in dendrites just activating its translation. Once expressed the GluN2B subunit it can assembly with the GluN1 subunit, which is synthesized in excess (estimated to be ≈ 10-fold) compared with GluN2 (Huh and Wenthold, 1999; Wenthold et al., 2003) forming active receptors. GluN2B mRNA 5'UTR contains 3 uAUG that represses the normal translation of GluN2B, as we showed by the uAUG reversion in the triple mutant.

NO is able to increase GluN2B expression and induces the phosphorylation of eIF2α by HRI. It links HRI to memory consolidation and learning processes in brain. But not only HRI is able to phosphorylate the eIF2α, so protein kinase RNA activated, double-stranded RNA-activated protein -like ER kinase, and general control non-derepressible 2 kinase can do it also. However, these kinases are related with stressful conditions and they would contribute to an increased GluN2B expression that would trigger glutamatergic excitotoxicity.

The functional assays by the measurement of Ca^{2+} showed that neurons treated with NO allowed a greater Ca^{2+} entry under NMDA plus Gly stimulation. This effect was prevented by the translational inhibitor, CHX, and for If, the specific GluN2B blocker. At synaptic level, NO increased the spontaneous response of the neurons and If,

blocked this increase, demonstrating the effect of GluN2B in synaptic response. The specific synaptic response was measured by BIC plus 4-AP, which blocks the γ-aminobutyric acid inhibitory response and depolarizes the neuron, enhancing its spontaneous activity in culture.

Summarizing, this work reports a novel regulation mechanism for GluN2B subunit expression in glutamatergic signalling, which can have important physiological implications in LTP and so, in memory and learning process.

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The authors declare no competing financial interests.

FIGURE 1

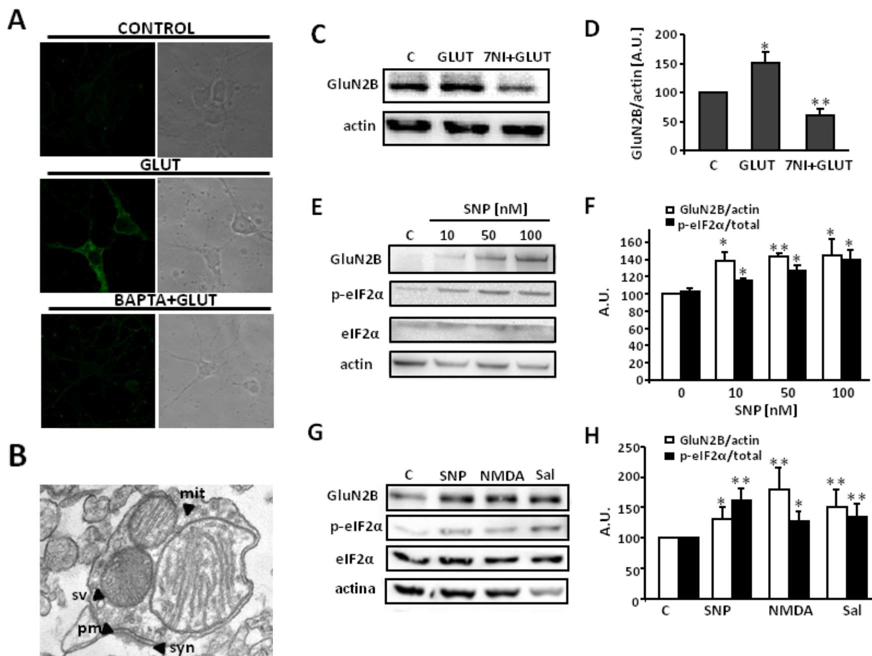

Figure 1. Glut increases GluN2B expression. (**A**) IF showing the increase of GluN2B subunit detection (green) in cultured mouse cortical neurons treated with 10 µM Glut for 1h in the absence and the presence of 10µM BAPTA·AM. (**B**) Electron microscopy image of a synaptosome prepared from mouse cortex; **mit**: mitochondria, **sv**: synaptic vesicles, **syn**: synapse, **pm**: post-synaptic membrane. (**C**) WB from cortical mouse synaptosomes treated with 10 µM Glut for 1h in the absence and the presence of 10 µM 7-NI. (**D**) WB band quantification regarding actin. Data are the mean ± SEM of 3 independent experiments.* $p<0.05$ and ** $p<0.005$ by Student T test. (**E**) WB of synaptosomes treated with increasing concentrations of SNP. (**F**) WB band quantification. Data are the mean ± SEM of 3 independent experiments. * $p<0.05$, ** $p<0.005$ by Student T test. (**G**) WB from cortical mouse synaptosomes treated with 100 nM SNP, 100 µM NMDA and 100 µM Sal. (**H**) WB band quantification. Data are the mean ± SEM of 5-20 independent experiments.* $p<0.05$ and ** $p<0.005$ by Student T test.

FIGURE 2

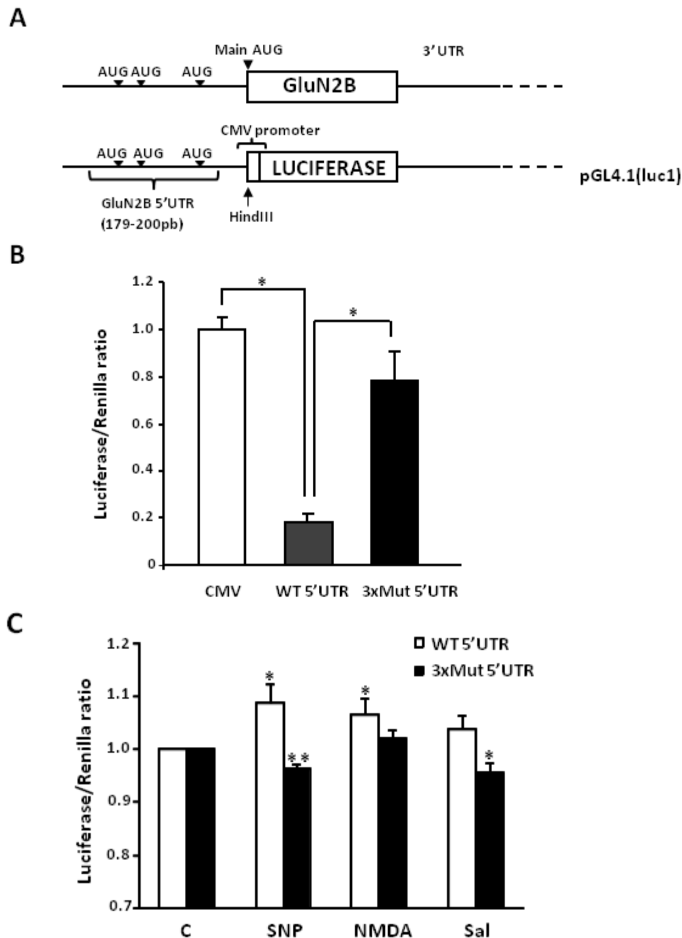

Figure 2. GluN2B translational repression by 5'UTR. (**A**) Scheme of the genomic structure of GluN2B showing the 5'UTR and the clonation of the 5'UTR in the pGL4.1 vector. The GluN2B 5'UTR is placed before the reporter gene luciferase and under the CMV promoter control. (**B**) Neuroblastoma cells were transfected with 25 ng of pGL4.1 vector with CMV, with the vector with the WT 5'UTR of GluN2B and with the vector with the mutation of the 3 ATGs 5'UTR of GluN2B. Data are the mean ± SEM of 3 independent experiments. * $p < 0.0005$ by Student T test. (**C**) Luciferase expression in neuroblastoma cells transfected with the vector with the WT 5'UTR and the 3x mut 5'UTR and treated with PBS (control), 100 nM SNP, 100 µM NMDA plus 100 µM Gly and 100 µM Sal for 1h. Data are the mean ± SEM of 3-4 independent experiments. * $p < 0.05$ ** $p < 0.005$ by Student T test.

FIGURE 3

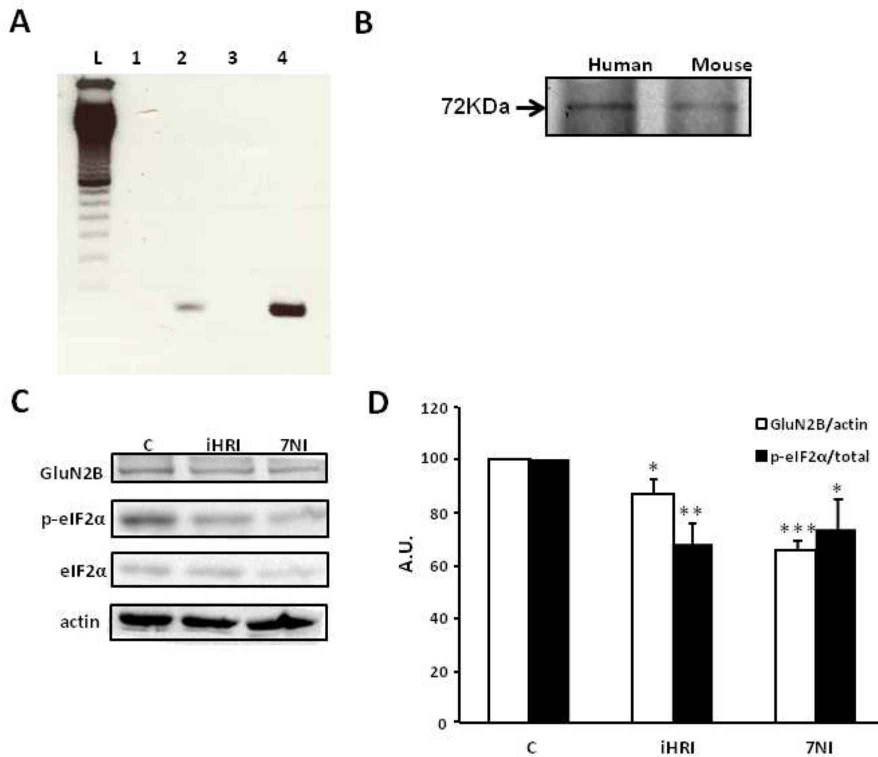

Figure 3. HRI kinase involvement in p-eIF2α and GluN2B translation. (**A**) PCR performed into 2% agarose gel. **L**: molecular weight ladder; **1**: human HRI primers without cDNA template; **2**: HRI amplification from cDNA of human cortex; **3**: mouse HRI primers without cDNA template; **4**: HRI amplification from cDNA of mouse cortical neurons. (**B**) HRI immunoprecipitated from human cortex and mouse cortical neurons. (**C**) WB of synaptosomes from mice trated with DMSO (control), 50 mg/Kg iHRI and 50 mg/Kg 7-NI. (**D**) WB band quantification. Data are the mean ± SEM of 4 independent experiments. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ by Student T test.

FIGURE 4

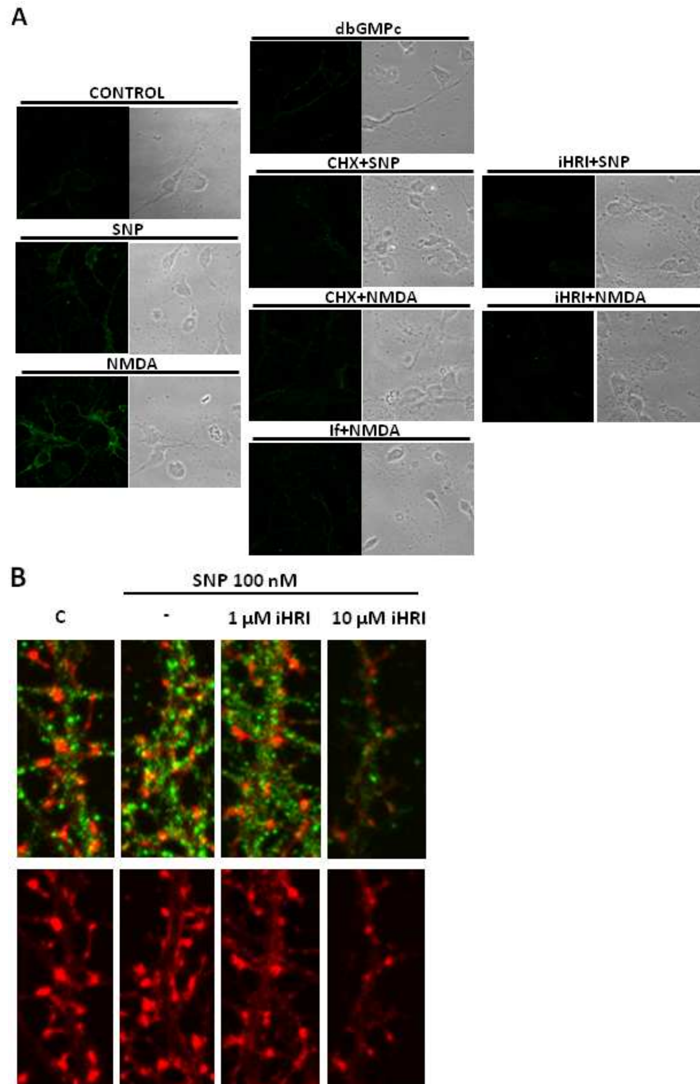

Figure 4. GluN2B expression on mouse cortical neurons and synaptic spines of rat cortical neurons. (**A**) Images of mouse cortical neurons treated with 100 nM SNP, 100 μM NMDA plus 100 μM Gly, 100μM CHX, 10 μM If, 1μM iHRI and 100μM dbGMPc for 1 h. GluN2B expression is shown in green. (**B**) Representative image obtained by immunofluorescence showing GluN2B expression (green) and phaloidin staining of actin (red) in synaptic spines. Synaptic spines were treated with SNP in the absence and the presence of iHRI.

FIGURE 5

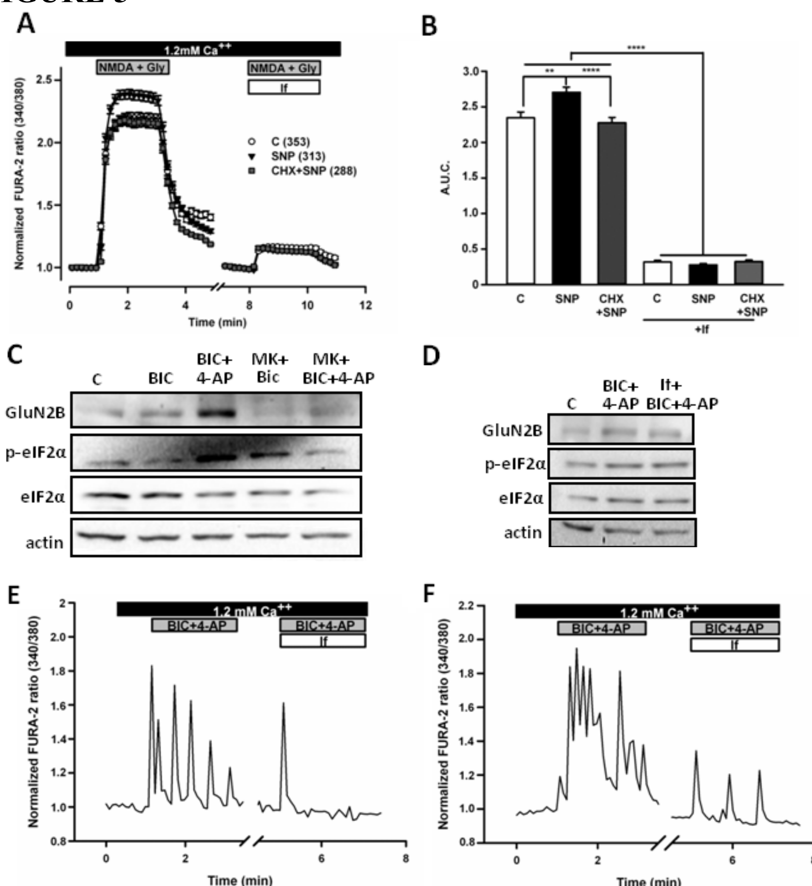

Figure 5. Effect of SNP in GluN2B mediated extrasynaptic and synaptic activity. (**A**) Measurement of Ca^{2+} entry in cortical neurons after stimulation with 100 µM NMDA plus 100 µM Gly and the 10 µM If plus 100 µM NMDA plus 100µM Gly for 2 min. Neurons were untreated (white circles) or treated with 100nM SNP (black triangles) and 100 µM CHX plus 100nM SNP (grey squares) for 1h. (**B**) Quantification of the AUC of each plot showed in A. Data are mean ± SEM of 288-353 cells from 6-7 independent experiments *p<0.005 **p<0.0005 by Student T test. (**C, D**) Representative WB showing the effect of 50 µM BIC and 50µM BIC plus 2.5 mM 4-AP for 1h. NMDA inhibitors MK-801 (**C**) and If (**D**) were also tested. **(E)** Representative plots showing the intracellular Ca^{2+} entry after BIC plus 4-AP and BIC, 4-AP plus If stimulation in cortical neurons untreated (**F**). Representative plots showing the intracellular Ca^{2+} entry after BIC plus 4-AP and BIC , 4-AP plus If stimulation in cortical neurons treated with SNP for 1h.

TABLE 1

Treatment	peaks/min	maximum peak
C	2.32 ±0.059	0.65 ±0.017
C+if	1.38 ± 0.083**	0.43± 0.011**
SNP	2.76 ± 0.065**	0.7± 0.015*
SNP+If	0.94 ± 0.078**	0.41± 0.007**

Peaks/min and Maximum peak in cortical neurons stimulated with BIC+4-AP
Data are mean ± SEM of 43-148 cells from 6 independent experiments, $^{*}p < 0.05$ $^{**}p < 0.00005$ by Student T test.

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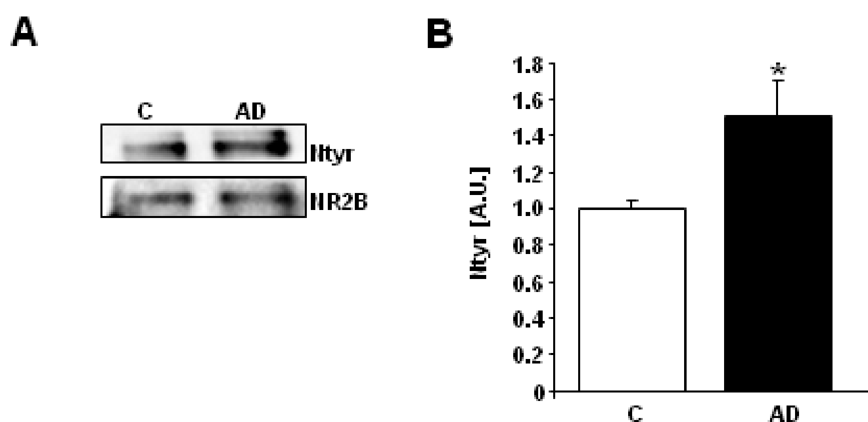
I. Addendum- figure 1.

The study of the nitrotyrosination (Ntyr) of NR2B subunit was performed in controls and AD patients demonstrating a higher Ntyr of the subunit in patients versus non-demented controls.



Addendum figure1. Study of the differential Ntyr of NR2B in cortex from controls and AD patients. (A) Western blot from immunoprecipitated NR2B from cortex of controls and AD patients. (**B**) WB band quantification. The Ntyr levels were corrected by the levels of the immunoprecipitated NR2B. Data are the mean ± SEM of 3 independent experiments $^{*}p<0.05$ by Student T test.

Matherials and methods addendum figure 1

Immunoprecipitation of NR2B from human brains

400 µg of homogenated brain were used for the immunoprecipitation. The samples were pre-incubated half an hour at 4ºC with protein G (GE Healthcare UL limited) which is previously washed with PBS. This step is needed to avoid unspecific protein binding with protein G. Then, the samples were centrifuged at 10,000 x g for 10 min. The SN was incubated

overnight (o.n.) with 5 μg of anti-NR2B Ab (Neuromab Abs). Following the addition of protein G immobilized on sepharose, samples were shaken for 2 h at room temperature (RT). NR2B was pulled down by centrifugation at 12,500 x g for 10 min and washed thrice. 60 μl of loading buffer x5 was added to pellet and it was boiling for 6 min at 100ºC. Immunoprecipitated NR2B was recovered by centrifugation at 12,500 x g for 10 min. 30 μL of this sample was resolved in 8 % polyacrilamide gel. Gels were transferred to polyvinylidene fluoride membranes (Immobilon-P transfer membranes). Membranes were blocked with 5%-tween Tris buffer saline (TTBS) 1x- 5% milk solution for 1h. Ntyr of NR2B was detected by incubating the membranes with 1:1000 mouse Ntyr antibody (Ab; Cayman) and NR2B Ab o.n. at 4ºC. Afterwards, three washes with TTBS 1x for 5 min were done. 1:4000 rabbit anti-mouse Ab (GE Healthcare UK) were used as secondary Abs at RT for 2 h to detect the primary Abs. Three washes with TTBS 1x for 5 min were performed and membranes were developed with the Supersignal West Femto Chemiluminiscent substrate (ThermoScientific). Band quantification was made with Quantity One. Ntyr detection was normalized by the amount of NR2B immunoprecipitated in each sample.

CHAPTER II:

Physiopathology of nitro-glycative modification of albumin in Alzheimer's disease

Physiopathology of nitro-glycative modifications of albumin in Alzheimer's Disease

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Running Title: Albumin nitro-oxidative modifications

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Summary

Alzheimer's disease (AD) is characterized by increased oxidative stress, which induces protein glycation. Furthermore, superoxide anion and nitric oxide can react to form peroxynitrite anion that damages proteins through tyrosine nitration, a process termed nitrotyrosination. All these protein modifications make them prone to losing their physiological properties. Albumin is the most abundant protein in blood and cerebrospinal fluid, where it carries out essential functions such as being a molecular carrier, maintaining oncotic pressure, scavenging harmful compounds, providing nutrients and, of particular relevance to our work, binding amyloid ß-peptide (Aß). In the present study we have analyzed albumin modification and function under the pro-nitro-oxidant conditions present in AD brains. We found that plasma and brain levels of nitrated and glycated albumins were significantly higher in AD patients than in controls. Turbidometry and electron microscopy analyses demonstrated that albumin nitrotyrosination and glycation promote changes in albumin structure affecting to its biochemistry. Glycated albumin, unlike nitrotyrosinated albumin, was more resistant to proteolysis with trypsin and its uptake by cells was reduced. Albumin glycation also caused a reduction in the osmolarity expected for a solution containing unmodified albumin. Moreover, modified albumin was cytotoxic in a cell type-dependent manner for cerebral and vascular cells. Furthermore, modified albumin binds more Aß than the native protein. In summary, nitrotyrosination and, more especially, glycation of albumin alter its

structural and physiological properties and these modifications could be relevant for the progression of AD.

Key Words: Albumin; Alzheimer's disease; amyloid ß-peptide; glycation; nitrotyrosination; oxidative stress.

Nonstandard abbreviations used : Alzheimer's disease (AD); Amyloid ß-peptide (Aß); antibody (Ab); Bicinchoninic Acid (BCA); Blood Brain Barrier (BBB); cerebral spinal fluid (CSF); fetal bovine serum (FBS); human aortic vascular smooth muscle cell line (HA-VSMC); human hepatocellular carcinoma cell line (HepG2); human neuroblastoma cell line (SH-SY5Y); human umbilical vein endothelial cell line (HUVEC); immunoprecipitation (IP); low-density lipoprotein receptor-related protein 1 (LRP-1); methylglyoxal (MG); Methylthiazolyldiphenyl-tetrazolium bromide (MTT); peroxynitrite donor (SIN-1); murine microglial cell line (BV2); nitrotyrosine (NT-3); overnight (o.n.); peroxynitrite ($ONOO^-$); phosphate buffered saline (PBS); Reactive Oxidative Species (ROS); room temperature (RT); supernatant (SN); Tween-tris buffer saline (TTBS); western blot (WB).

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease in aging people. It is characterized by increased oxidative stress in the brain as a result of the accumulation of extracellular amyloid beta-peptide (Aß). Aß aggregates produce cytotoxic oligomers and fibrils (Lambert *et al.*, 1998; Ill-Raga *et al.*, 2010) and finally forms the senile plaques found in brain parenchyma. Aß can also form vascular amyloid deposits, leading to cerebral amyloid angiopathy, which mainly involves small arteries and capillaries of the meninges, cerebral and cerebellar cortex in over 80% of the AD brains (Jellinger, 2002).

Aß aggregates produce reactive oxygen species (ROS) (Huang *et al.*, 1999) which, due to their gaseous nature can diffuse into the surrounding tissues, including vessels. This diffusion process is also favored by the subsequent amplification of free radical cascades (Zhou *et al.*, 2010). The superoxide anion reacts with nitric oxide, mainly produced by glial and endothelium cells (Boje & Arora, 1992; Coma *et al.*, 2005), to render peroxynitrite ($ONOO^-$). This peroxynitrite nitrates the tyrosine residues in proteins, a process termed nitrotyrosination, and dramatically affects their function (Crow & Beckman, 1995; Guix *et al.*, 2009). Glycation is another mechanism of oxidative modification of proteins that can be mediated directly by methylglyoxal (MG), a toxic compound overproduced by dysregulation of the glycolytic flow (Vitek *et al.*, 1994; Guix *et al.*, 2009), or indirectly produced through a Maillard reaction. This non-enzymatic reaction consists of the binding of

sugars to proteins, forming Amadori products, which facilitate the crosslink between glycated proteins (Monnier & Cerami, 1981).

Albumin is the most abundant protein in both blood and cerebrospinal fluid (CSF). The albumin present in the plasma is produced by the liver and a small fraction of plasmatic albumin enters the brain (Prajapati *et al.*, 2011) although most of the albumin found there is produced endogenously by the glia (Ahn *et al.*, 2008). It participates in the regulation of blood and CSF volume by maintaining the oncotic pressure. Albumin also transports different molecules like hormones, free fatty acids, calcium, other ions and some drugs. Albumin can also buffer oxidative damage due to the presence of free cysteine that is not forming disulfide bridges (Era *et al.*, 1995). Interestingly, albumin has been previously reported to bind Aß (Biere *et al.*, 1996) that could be favor amyloidogenic clearance by the liver. Aß40 is the most abundant form of Aß circulating in plasma (Smith & Betteridge, 2004) so it could have more importance in albumin binding than Aß42. In addition, albumin has a potential role as a molecular chaperone, preventing the misfolding and aggregation of proteins (Marini *et al.*, 2005) and specifically inhibiting Aß fibril formation (Bohrmann *et al.*, 1999).

Albumin has 19 tyrosines susceptible to modification by peroxynitrite (Ghesquiere *et al.*, 2006) as well as a high number of lysines, which are also quite prone to glycation (Ledesma-Osuna *et al.*, 2008). Both circulating and endogenous albumin can be affected by the increased nitro-oxidative and glycative stress present in ageing and age-associated diseases like AD (Smith *et al.*, 1991;

Golubev, 1996). Nitrotyrosination and glycation have been previously reported to induce protein aggregation (Guix *et al.*, 2009; Panza *et al.*, 2010). Under normal conditions, the low-density lipoprotein receptor-related protein-1 (LRP-1) releases Aß from the brain into the bloodstream through the blood brain barrier (BBB) (Shibata *et al.*, 2000). Albumin interacts directly with the neurons and endothelium, vascular smooth muscle and glia cells on both sides of the BBB (Prajapati *et al.*, 2011; Zoellner *et al.*, 2009; Lau *et al.*, 2011; Karmakar, 2001). However, systemic Aß circulating in plasma may also enter the brain in AD patients via the receptor for advanced glycation end products (Mackic *et al.*, 1998). This means that the hepatic clearance of albumin-bound Aß should reduce the level of circulating Aß and its presence in the brain (Ahn *et al.*, 2008; Carro *et al.*, 2002). In addition, some groups report that albumin inhibits Aß fibril formation (Bohrmann *et al.*, 1999), highly relevant to preventing Aß misfolding and aggregation.

This study analyses the albumin modifications caused by nitro-glycative stress. It also evaluates the presence of these modifications in the brain and plasma of AD patients, how these modifications affect albumin function, and their impact on different cell types present on both sides of the BBB.

Experimental Procedures

Cell lines

The HUVEC was cultured in M-199 medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM of L-Glutamine (Sigma-Aldrich), 100 UI/mL of penicillin and 100

μg/mL of streptomycin (Sigma-Aldrich). The HA-VSMC was cultured with MCDB 131 medium (Gibco BRL, Invitrogen), supplemented with 5% FBS, 0.5 ng/mL of epidermal growth factor (Sigma-Aldrich), 2 ng/mL of basic fibroblast growth factor (bFGF; Invitrogen), 5 μg/mL of insulin (Gibco BRL, Invitrogen), 2 mM of L-Glutamine and antibiotics. The BV2 cell line was cultured with RPMI 1640 medium (Gibco BRL, Invitrogen) supplemented with 10% FBS and antibiotics. The SH-SY5Y was cultured in Dulbecco's modified Eagle medium (Gibco BRL, Invitrogen) supplemented with 15% FBS and antibiotics. The human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and antibiotics.

Human brain samples

Human brain tissue samples were supplied by the Neurological Tissue Bank (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona), the Department of Pathology (Hospital del Mar, Barcelona) and the Neuropathology Unit and Brain Bank (Fundación Hospital Alcorcón, Madrid). The procedure was approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Brain samples were obtained from the frontal cortex of 13 healthy aged male and female individuals (mean ± SEM of 71±2 years) and 18 male and female AD patients at stage IV-VI (mean ± SEM of 78±3 years). The samples were lysated with a cocktail containing NP40 lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.05%

aprotinin, 1 mM dithiothreitol) and protease inhibitors (Complete mini-EDTA free) from Roche Diagnostics GmbH. Lysates were mechanically disaggregated using plastic micropestles (Eppendorf) and 1 mL syringes and the obtained brain tissue solution was centrifuged at 12,500 rpm for 10 min. The supernatant (SN) was quantified by the Bicinchoninic Acid assay (BCA; Pierce® BCA Protein assay kit, Thermo Scientific).

Human plasma samples

Plasma samples were obtained from 14 healthy elderly males and females (mean ± SEM of 70±1 years) participating in a population-based survey (Grau *et al*., 2007), and from 19 male and female AD patients (mean ± SEM of 74±2 years) diagnosed with mild to moderate AD (NINCDS-ADRDA criterion) and an Mini Mental State Examination score between 20 and 24. All the procedures were approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. All individuals, close relatives or legal representatives signed the corresponding informed consent before participation. Plasma samples were stored at -80 ºC.

Albumin nitrotyrosination and glycation

Unless indicated otherwise, 1.25 µg/µL of human albumin (Grifols) was incubated with increasing concentrations (100 µM to 50 mM) of the peroxynitrite donor SIN-1 (Sigma-Aldrich) and the glycative agent MG (CosmoBio Co., LTD) in a phosphate buffer solution (PBS). The solutions were stirred (300 rpm) for 3 hours at

room temperature (RT). After treatment, the albumin was filtrated by centrifugation at 11,000 rpm for 10 min in 30 KDa desalting filters (Vivacon 500 and Ultrafree-MC microcentrifuge filters, Sigma-Aldrich). Protein was recovered by revert spinning at 2,500 rpm for 2.5 min and quantified using the BCA.

Albumin immunoprecipitation (IP)

IP used 200 µL of plasma sample and 400 µg of homogenized brain. Samples were pre-incubated for half an hour at 4 ºC with G protein (GE Healthcare UK limited) previously washed with PBS. This step is required to avoid unspecific G protein binding. The samples were then centrifuged at 10,000 rpm for 10 min. The SN was incubated overnight (o.n.) with 5 µg of anti-albumin antibody (Ab; Acris Antibodies). Following the addition of sepharose-immobilized G protein (GE Healthcare UK limited), samples were shaken for 2 hours at RT. Albumin was precipitated by centrifugation at 10,000 rpm for 10 min and washed three times. 60 µl of loading buffer x5 were added to the pellet and the mix was boiled for 6 min at 100 ºC. The boiled samples were centrifuged at 10,000 rpm for 10 min and 30 µL of this SN was resolved in 8% polyacrylamide gel. Gels were transferred to polyvinylidene fluoride membranes (Immobilon-P transfer membranes) and the nitrotyrosination and glycation detection was performed as described below. A stripping solution was applied to membranes to wash the previous Abs (three washes with PBS-0.05% Tween; incubation for 30 min at 80 ºC with Glycine 0.2 M at pH 2.5; and three more washes with PBS-Tween).Membranes were then incubated with anti-albumin Ab in a 1:1000 dilution with tween tris

buffer saline (TTBS)-5% milk o.n. at 4 ºC and they were developed for MG and nitrotyrosine (NT-3) detection as explained below. Band quantification was performed using Quantity One from BioRad and the MG and NT-3 detection normalized by the amount of albumin in each sample.

Nitrotyrosination and glycation detection by WB

18.75 µg of unmodified, glycated or nitrated albumin were resolved in 8% polyacrylamide gels. Gels were transferred to polyvinylidene fluoride membranes. Membranes were boiled for 5 min before the glycation study and then blocked with TTBS-5% milk solution for 1 hour. Nitration and glycation were detected by incubating the membranes with 1:1000 mouse NT-3 Ab (Cayman) and 1:1000 mouse anti-argpyrimidine Ab (CosmoBio Co, LTD) o.n. at 4ºC, followed by three washes with TTBS for 5 min. Rabbit anti-mouse secondary Ab (1:4000, GE Healthcare UK limited) was used at RT for 1 hour. The membranes were washed three times with TTBS for 5 min and developed with the SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) in the ChemiDoc MP imaging system (Bio-Rad). Blotting quantification was performed with the ImageJ (NIH) program.

Aggregation assays

Turbidimetric assays were performed with 10 µg/µL of albumin in PBS (control) and plus 25 mM SIN-1 for nitrative assays or with 25 mM MG for glycative assays. The assays were carried out in Nunc 96-well plates stirred (300 rpm) at RT for up to 24 hours.

Absorbances were measured at 405 nm in the Tecan Infinite M200 spectrophotometer.

Osmolarity assays

30 μg/μL of albumin were incubated with 100 μM, 12.5 mM or 25 mM of SIN-1 or MG and stirred (300 rpm) at RT for 48 hours. Sample osmolarity was measured with an osmometer (Fiske One-ten osmometer).

Transmission electron microscopy images

1.25 μg/μL albumin were treated with 12.5 mM SIN-1 or 50 mM MG for 4 weeks with continuous stirring (300 rpm) at RT. Nickel mesh grids were charged with ultraviolet light for 5 min and set on a drop of sample (with 0.625 μg/μL of albumin) for 1 min. They were then washed three times with milliQ water (1 min in total). Finally the grid was set on a drop of 2% uranyl acetate solution for 1 min and dried. Samples were observed with a Jeol 1010 electron microscope.

Albumin degradation by trypsin

Unmodified, nitro- and glyco-albumin were incubated with 0.05% Trypsin-EDTA (Gibco BRL, Invitrogen) for 24 hours. A soy trypsin inhibitor (STI; Sigma-Aldrich) was used at 1 mg/mL to stop trypsin activity. Albumin samples were resolved in 8% polyacrylamide gels. Protein detection used Coomassie protein staining techniques.

Albumin uptake by HepG2 cells

HepG2 cells were seeded in 6-well plates at a density of 7.5 x 10^5 cells/mL/well. Unmodified, nitro- and glyco-albumin at 30 μg/μL

were then added. The medium was withdrawn after 24 hours and the albumin concentration was measured by BCA and Bradford protein quantification method (Bio-Rad protein assay).

Cell viability assays

HUVEC, HAVSMC, BV2 and SH-SY5Y cells were seeded in 96-well plates in their respective media at a density of 10^4 cells/100µL/well. Cells were incubated with PBS (controls), untreated, nitrotyrosinated, and glycated albumin at 1 µM (0.068 µg/ µL, in the physiological concentration range present in CSF), 500 µM (34.5 µg/µL, the physiological concentration in plasma), and SIN-1 and MG in PBS at the respective concentrations used for albumin treatments. Cells were treated for 24 hours at 37ºC. Cell viability was measured by methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction. Briefly, 10 µL of MTT (Sigma-Aldrich) stock solution (5 mg/mL) were added and after 2 hours the reaction was stopped with 100 µL of dymethylsulfoxide. MTT was determined in a plate reader spectrophotometer (FLUOstar optima, BMG labtech) at 540 and 650 nm. Control cells were taken as 100%.

Aß aggregation assays

0.1 mg of human Aß40 (Sigma-Aldrich) was diluted in 67 µL of dymethylsulfoxide. 33.5 µL of soluble Aß were taken to 750 µL with PBS and stirred (300 rpm) at RT for 3 days in order to produce Aß fibrils and 1 hour to produce oligomers.

Aß binding assays

30 µg/µL of unmodified, nitro- and glyco-albumin were incubated with 180 pg/µL of soluble and aggregated Aß40 for 24 hours. These samples were incubated with 5 µg of anti-albumin monoclonal Ab (Acris Antibodies) o.n. at 4 ºC. Following the addition of sepharose immobilized protein G (GE Healthcare UK limited), samples were shaken for 2 hours at RT. Aggregates were precipitated by centrifugation at 10,000 rpm for 10 min and washed three times. Protein G and Ab were removed from the immunoprecipitated proteins by boiling the samples for 6 min at 100 ºC. The SN containing the immunoprecipitated albumin together with the bonded Aß, were added in the Human Amyloid ß assay kit (Immuno-Biological Laboratories Co., LTD) and the amount of Aß measured.

Statistical analysis

Data are expressed as the mean ± SEM of the values from the number of experiments as indicated in the corresponding figures. Data was statistically evaluated using Student's *t*-test.

Results

Albumin is nitrotyrosinated and glycated in human samples from AD patients

The presence of nitro- and glyco-albumin in the plasma and brains of AD patients and aged non-demented controls is shown in Fig. 1. Increased albumin nitrotyrosination (Fig. 1A) was found in both

plasma ($p<0.01$) and brain samples ($p<0.05$) from AD patients. Similar results were found regarding albumin glycation (Fig. 1B). Significant increases in glycated albumin were also detected in both plasma ($p<0.05$) and brain samples ($p<0.05$) from AD patients (Fig. 1B).

Albumin is nitrotyrosinated and glycated in a concentration-dependent manner by peroxynitrite and methylglyoxal

The ability of albumin to be nitrated by SIN-1, a peroxynitrite donor, and glycated by MG, a glycative agent, measured by western blot (WB) analysis, is shown in Fig. S1. Albumin nitration augmented with increasing doses of SIN-1 up to 12.5 mM. A SIN-1 concentration of more than 12.5 mM did not produce greater nitration probably due to complete nitration of all the tyrosine at this concentration (Fig. S1 A and B). Albumin glycation shows a different kinetic pattern, it augmented with increasing MG concentration up to 7.5 mM whereas higher doses of MG triggered “an apparent decrease” of albumin glycation (Fig. S1 C and D). This effect at concentrations above 7.5 mM MG could be due to cross-linking of glycated albumin to form large aggregates unable to enter the polyacrylamide gels and, therefore, not be detected at the molecular weight of monomeric albumin.

$ONOO^-$ and MG induce albumin aggregation in vitro

Nitrotyrosination and glycation have been previously reported to induce protein aggregation. Turbidimetric assays consistently showed aggregation of nitro- and glyco- albumin compared to

untreated albumin (Fig. 2A and C). The aggregation of nitro-albumin showed higher turbidimetry absorbance values than glyco-albumin. Not only are the aggregation kinetics between nitrated and glycated albumin different, but the appearance of the aggregates differ as well. Structural differences between nitrotyrosinated and glycated albumin aggregates were evident under electron microscopy after 4 weeks of treatment (Fig. 2B and D). Nitro-albumin aggregates were more condensed that untreated and glycated albumin aggregates. In addition, glyco-albumin aggregates showed globular structures different to those present in untreated or nitrated albumin aggregates. The turbidimetry pattern observed for glyco-albumin could be due to the formation of larger and more expanded aggregates as this would explain the results obtained by WB and electron microscopy.

Trypsin digestion and cell uptake of nitro- and glyco-albumin

Albumin turnover, taking approximately 21 days in humans (Bennhold & Kallee, 1959) involves its degradation and uptake by liver cells, a process that may be affected by its nitration and glycation. To address this point, an evaluation was made of the digestion of modified and unmodified albumin. Untreated, nitro- and glyco-albumin samples incubated with trypsin are shown in Fig. 3A. As expected, higher degradation (fragments <50 kD molecular weight) was observed in untreated albumin compared to nitro- and glyco-albumin. Digestion of nitro-albumin produced less low molecular weight fragments. Glyco-albumin was the most resistant to trypsin digestion. Next, an assessment was made of the cellular

uptake of modified and unmodified albumin by hepatoma cells (Fig. 3B). In cells incubated with unmodified, nitro- and glyco-albumin in a serum-free medium, there was significantly glyco-albumin uptake (0.415 ± 0.331; $p<0.05$) by cells compared to untreated albumin (6.899 ± 3.315) whereas nitrotyrosinated albumin uptake was not affected (6.048 ± 4.038).

Albumin glycation reduces osmolarity

Since nitrotyrosination and glycation produce albumin aggregates, these modifications were studied to determine whether they could also be affecting osmotic pressure. Measurements were made of the osmolarity of solutions containing the same concentrations of untreated albumin, nitro-albumin and glyco-albumin (Fig. 4). Nitrotyrosination of albumin with SIN-1 did not affect osmolarity (Fig. 4A) whereas albumin glycation with increasing concentrations of MG (Fig. 4B) showed significant ($p<0.05$) reduction in osmolarity compared to control albumin. This reduction in osmolarity is probably due to trapping of the ions dissolved in the medium.

Effect of albumin nitrotyrosination and glycation on cell viability

Albumin interacts directly with the neurons, endothelium, vascular smooth muscle and glia cells on both sides of the BBB (Prajapati *et al.*, 2011; Zoellner *et al.*, 2009; Lau *et al.*, 2011; Karmakar, 2001). The effect of albumin glycation and nitration on cell viability was tested using endothelial (HUVEC), vascular smooth muscle (HA-VSMC), glial (BV2) and neuronal (SH-SY5Y) cell lines. The

results for cells incubated with the physiological concentration of albumin in plasma (500 µM) and the albumin concentration range present in CSF (1µM), nitro-albumin, and glyco-albumin are shown in Fig. 5A and Fig. 5B, respectively. Deleterious effects of glyco-albumin are observed in glial and neuronal cell lines while the most toxic effect of nitro-albumin is on glial and smooth muscle cells. HUVEC are the most resistant cells to either the nitrotyrosinating and glycating agents alone or the modified albumins.

Increased Aß binding to nitro- and glyco-albumin

The results of Aß binding experiments carried out with soluble and fibrillar Aß40 are shown in Fig. 6. The binding assay showed that nitro-albumin and glyco-albumin bind more Aß40 in soluble form than control albumin ($p<0.005$; $p<0.05$). Furthermore, glyco-albumin binds more Aß40 in aggregated form than control albumin ($p<0.05$).

Discussion

High nitro-oxidative stress is associated with the AD brain (Smith *et al.*, 1991; Thorns *et al.*, 1998; Huang *et al.*, 1999; Kuhla *et al.*, 2005; Guix *et al.*, 2009) and this could affect the proteins on both sides of the BBB because of the gaseous nature of ROS and the chain reactions induced by these species in surrounding tissues. Albumin is the most abundant protein in both blood and CSF, in addition to its physiological functions, albumin may also play a major role as a scavenger of ROS, favored by its high turnover with

a life of 21 days (Bennhold & Kallee, 1959). This process involves its degradation and uptake by liver cells and may be affected by albumin nitration and glycation. However, this ability might have consequences for albumin when the buffering threshold for oxidative stress is exceeded, as occurs in AD. This study focused on albumin nitrotyrosination and glycation, and how these modifications affect its properties.

Both nitrotyrosination and glycation make albumin more prone to aggregation. In the case of nitrotyrosination, the interaction of nitrated tyrosines from several molecules of albumin-producing dityrosine bridges could lead to its aggregation (Foerder & Shapiro, 1977). Albumin glycation could also induce aggregation probably due to cross-linking between ketone groups from Schiff bases, the intermediate products after protein glycation (Cerami *et al.*, 1987). The observed “apparent decrease” effect at concentrations higher than 7.5 mM MG could be due to the cross-linking of glycated albumin forming large aggregates unable to enter the polyacrylamide gels and, therefore, not being detected at the molecular weight of monomeric albumin. The changes in modified albumin observed under the transmission electron microscopy showed denser aggregates in nitrotyrosinated albumin and more globular aggregates in glycated albumin than those observed in unaltered albumin. These results are consistent with increased albumin aggregation.

The data suggest that these stable aggregates of modified albumin are not easily broken down, and therefore additional, accessory proteins may be required for their degradation (Friguet *et al.*, 2000;

Shringarpure & Davies, 2002). Glyco-albumin was the most resistant to trypsin digestion, and this is consistent with previous reports showing that protein glycation reduces their normal degradation (Brownlee *et al.*, 1983). Besides, as proteolysis of large aggregates is more difficult, modified albumin will be circulating for longer periods of time, affecting its turnover and reducing its protective role. Another consequence of albumin modification affects maintenance of osmotic pressure. Compared to unmodified albumin, the presence of glycated, but not nitrotyrosinated albumin, significantly decreases the osmolarity of the solution it is dissolved in. This effect might have deleterious consequences, especially for small brain vessels where it will favor plasma extravasation (Fleck *et al.*, 1985) and homeostasis of abnormal tissue.

We also found that nitro- and glyco-albumin decrease the viability of vascular myocytes, glia cells and neurons, while endothelial cells appear to better tolerate the presence of modified albumin. HUVEC cells were the most resistant to nitrotyrosinating and glycating agents alone or modified albumins. This difference may indicate that a particular albumin modification will mainly affect a particular set of cell types. Since both nitrative and glycative modifications are additive *in vivo,* their deleterious effects would preferentially affect neurons, glia cells and vascular myocytes. Another interesting observation is that albumin may act as a buffer for the nitro-oxidative and glycative stress since the presence of albumin reduced the toxic effect of equimolar concentrations of SIN-1 and MG alone. The reduction in cell viability could be due to a lack of the nutritive properties of albumin (Kirsch *et al.*, 1968) or other

mechanisms derived from its aggregated structure. Interestingly, nitrating and glycating agents are always more toxic to cells than the corresponding concentration of modified albumin, reinforcing the idea that albumin protects against these reactive species by buffering them (Bar-Or *et al.*, 2001).

Our results show that nitro- and glyco- albumin binds more Aß40 than native albumin does and this could be related to the ability of nitro- and glyco-albumin to interact with highly hydrophobic Aß (Bouma *et al.*, 2003; Du & Murphy, 2010). In the first instance, higher Aß binding to modified albumin could be beneficial for AD patients, since it would remove Aß from the brain through low-density LRP-1 (Shibata *et al.*, 2000). But considering that modified albumin may have a longer plasma life than normal albumin due to its resistance to degradation, in the long run, the ability to bind Aß would be decreased after saturation of the existing modified albumin, thus allowing systemic Aß to enter the brain through receptor for advanced glycation end products (Mackic *et al.*, 1998). Therefore, modified albumin cannot buffer more pro-oxidant challenges, and consequently, also fails in protecting the brain against nitro-oxidative stress. In fact, some studies demonstrate that peripherally derived Aß in circulating plasma represents an important precursor pool for brain Aß (Eisele *et al.*, 2010) since it can cross the BBB in both directions depending on the Aß concentrations in brain and blood. It is possible that improvements in the cognitive scores of AD patients treated by albumin replacement described in a previous report (Boada *et al.*, 2009) is related to the removal of nitro- and glyco-albumin.

In conclusion, nitrotyrosination and glycation favors albumin aggregation. These processes, especially glycation, affect albumin physiological functions, including increased Aß40 binding capacity. Further studies should be carried out to evaluate the possibility of this phenomenon being related to the beneficial effect of albumin replacement therapy in AD patients.

Acknowledgments

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FIGURE 1

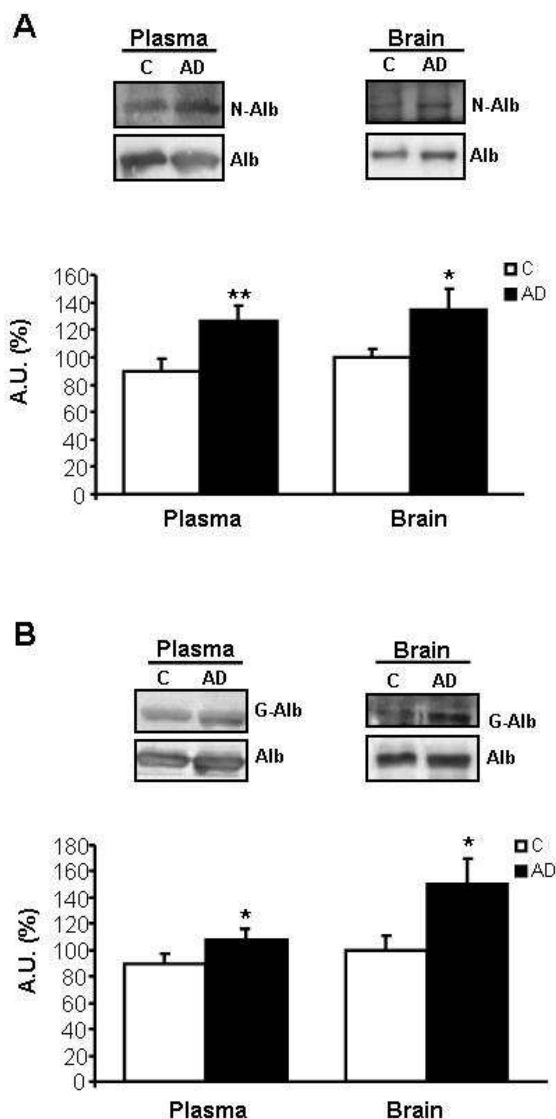

Figure 1. Nitrotyrosinated and glycated albumin in human samples from AD patients. Nitrotyrosinated (A) and glycated (B) albumin was quantified by WB after obtaining albumin by IP from plasma (18 controls and 19 AD) and brain samples (13 controls and 18 AD). Data are mean ± SEM of the number of experiments indicated between brackets. $*p<0.05$ for all experiments except the nitrotyrosination in albumin from plasma ($**p<0.01$).

FIGURE 2

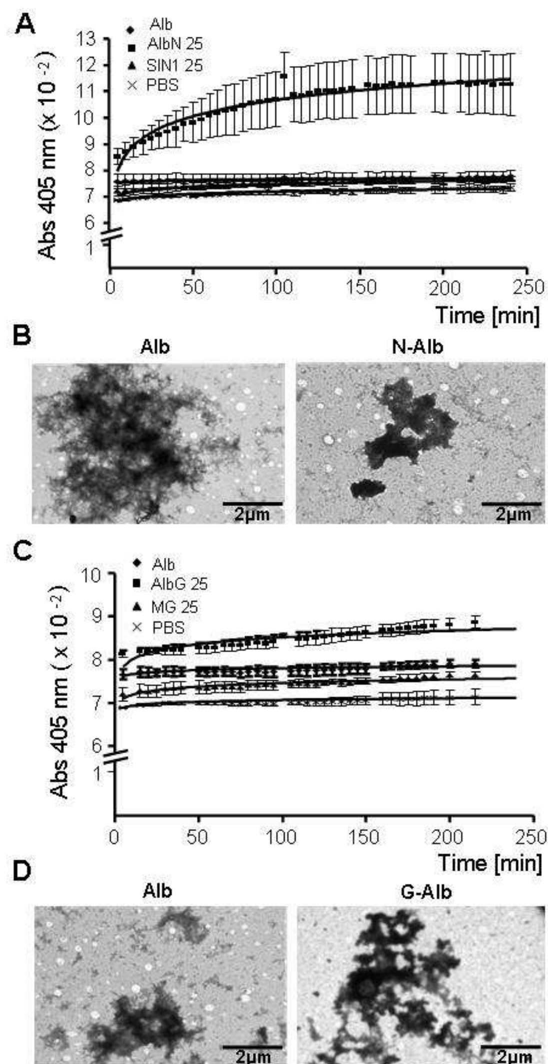

Figure 2. $ONOO^-$ and MG induce albumin aggregation. Turbidimetric assays showed increased nitro- (**A**) and glyco-albumin (**C**) aggregation compared to untreated control albumin. Transmission electron microscopy images of untreated albumin (**B**, **D**), nitro- (**B**) and glyco-albumin (**D**). Data are the mean ± SEM of 3 independent experiments.

FIGURE 3

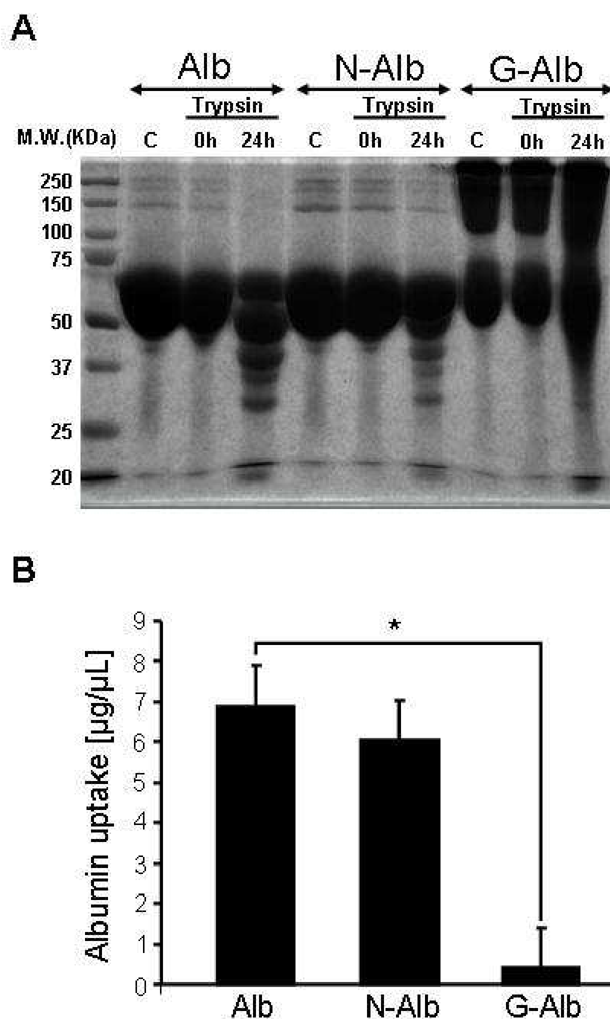

Figure 3. Trypsin digestion and uptake of nitro- and glyco-albumin into HepG2 cells. Unmodified, nitro- and glyco-albumin were incubated with trypsin at different times. The pattern of degradation was detected by Coomassie staining (n=4) (**A**). Unmodified, nitro- and glyco-albumin uptake in HepG2 cells was studied after incubation for 24 hours (**B**). Data are the mean ± SEM of 3-5 independent experiments. * $p < 0.05$.

FIGURE 4

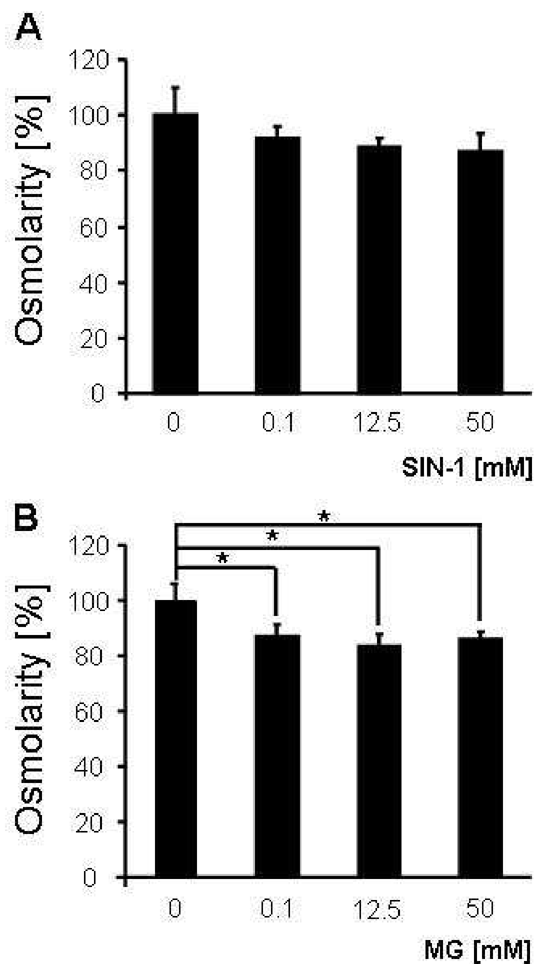

Figure 4. Impact of albumin glycation in osmolarity. The effect of nitrotyrosination (**A**) and glycation (**B**) of albumin on the osmolarity of solutions was studied *in vitro*. Data are the mean ± SEM of 3-6 independent experiments. * $p < 0.05$.

FIGURE 5

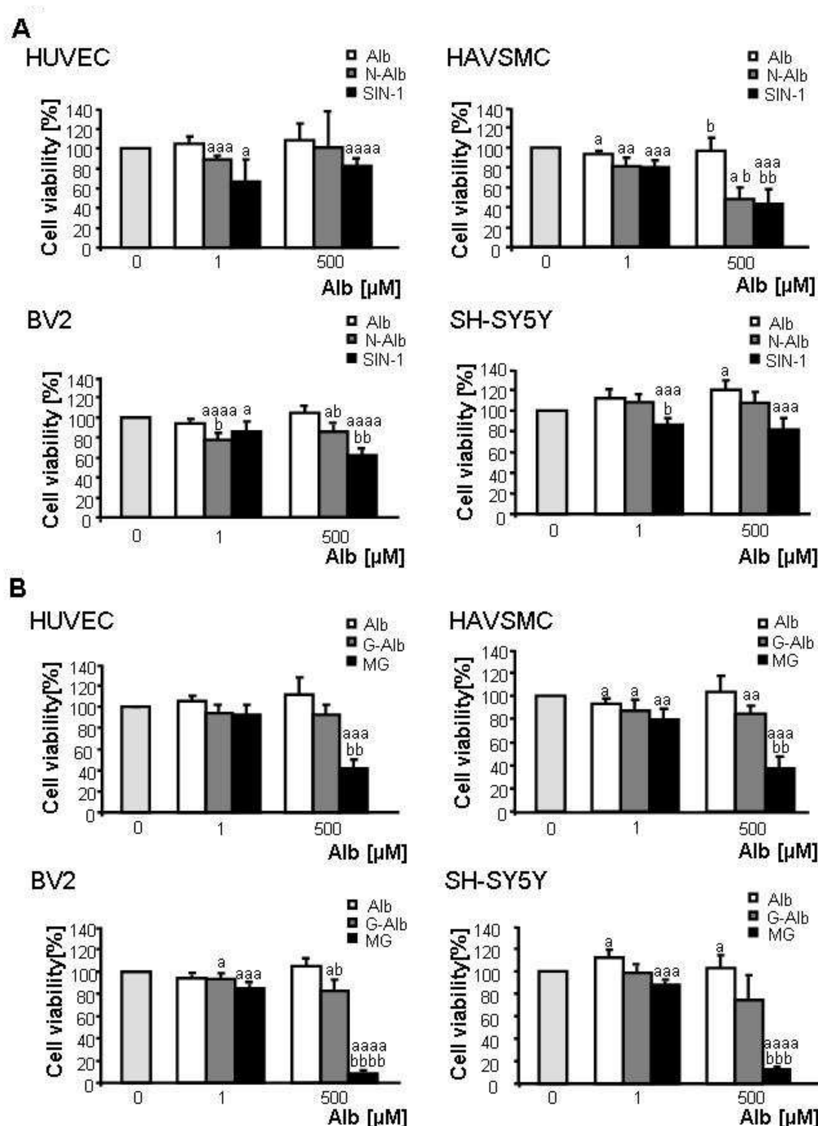

Figure 5. Effect of nitro-albumin and glyco-albumin on cell viability. Vascular (HUVEC and HA-VSMC), glial (BV2) and neuronal (SH-SY5Y) cells were treated with nitro-albumin (**A**) or glycol-albumin (**B**) for 24 hours. Cell viability was measured by MTT reduction. Data are the mean ± SEM of 4-6 independent experiments performed in triplicate. [a] $p<0.05$ (treated *vs.* control); [b] $p<0.05$ (N-Alb or G-Alb and SIN-1 or MG *vs.* Alb).

FIGURE 6

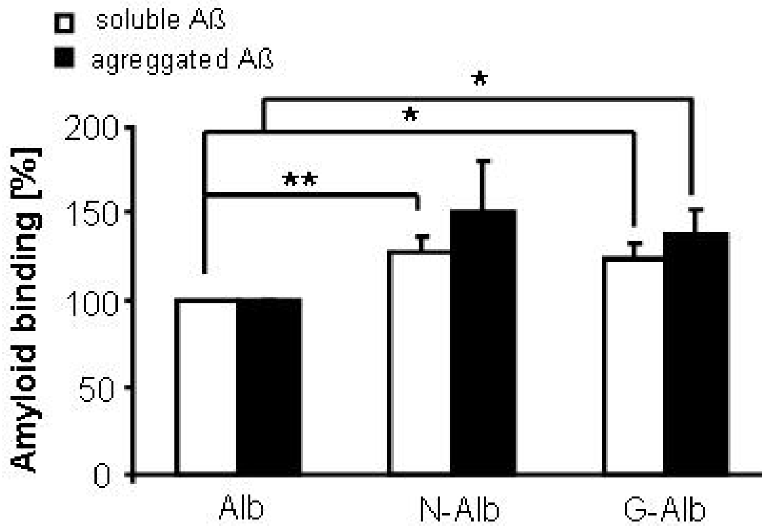

Figure 6. Binding of Aß to nitro- and glyco-albumin. Synthetic aggregated and soluble Aß40 were incubated with unmodified, nitro- and glyco-albumin for 24 hours. The Aß binding was measured by an ELISA kit. Data are the mean ± SEM of 4 independent experiments. * $p < 0.05$.

SUPPLEMENTARY FIGURE

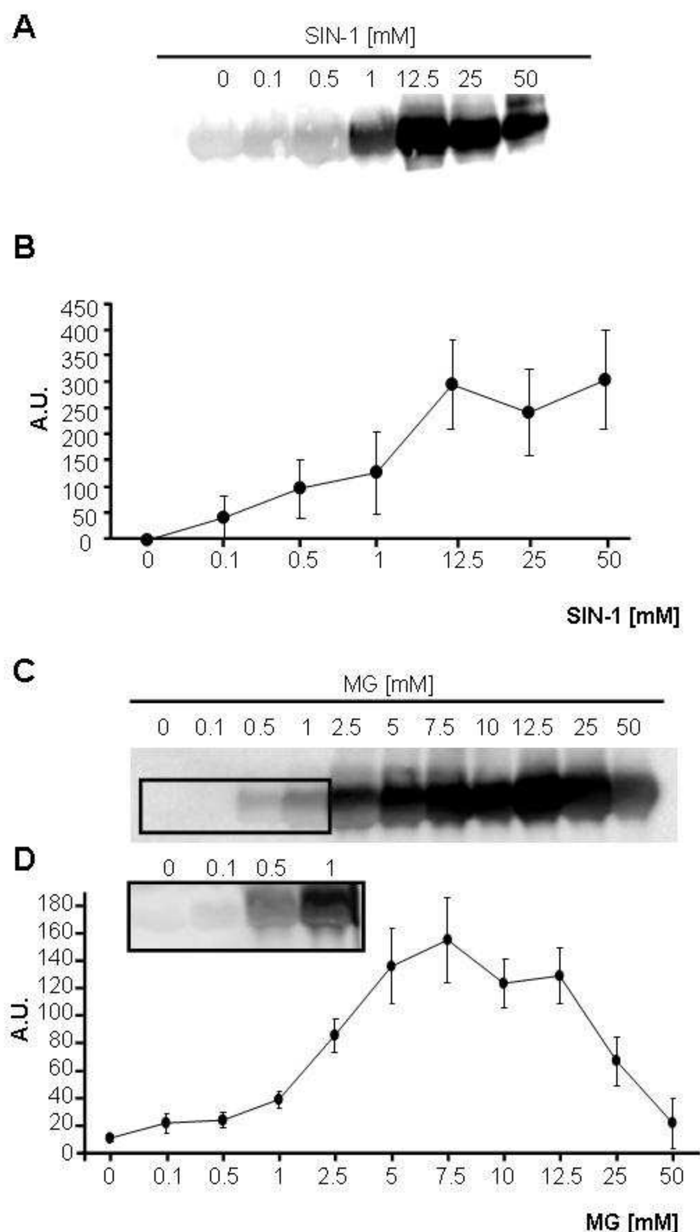

Figure S1. Concentration dependent albumin nitrotyrosination and glycation by ONOO- and MG.

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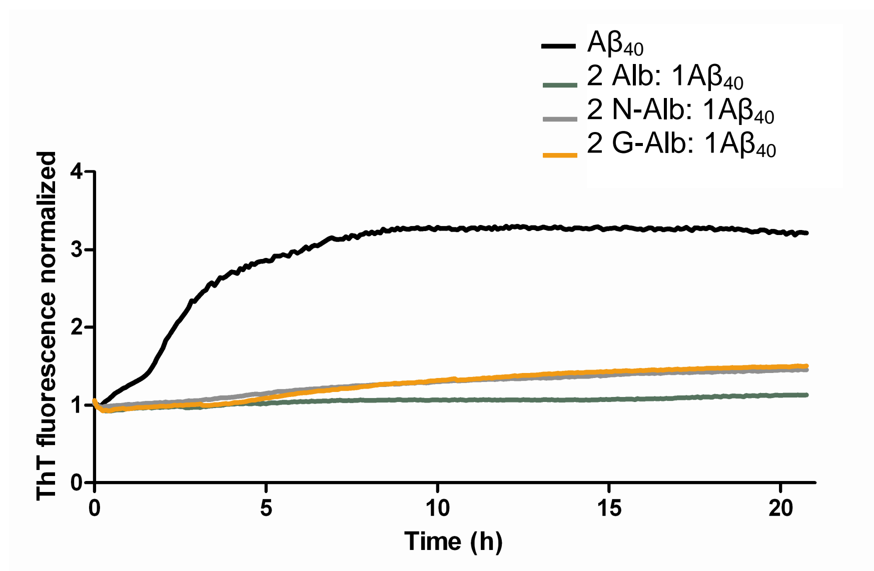
II. Addendum –figure 1.

Measurement by ThT fluorescense of the differential Aβ40 aggregation in presence of albumin or modified albumin. Albumin is able to abolish completely Aβ40 aggregation, but nitro- and glyco-albumin reduces the albumin capability of inhibits Aβ40 fibril aggregation.



Addendum figure1. Study of the differential Aβ40 fibril aggregation with the co-incubation of albumin, nitro-albumin and glyco-albumin by ThT fluorescence measure. Data are the mean ± SEM of 3-4 independent experiments.

Matherials and methods addendum figure 1

ThT assay

ThT assay is based on the ThT property to bind β sheet rich structures, such as those amyloid aggregates, and display enhanced fluorescence and a characteristic red shift of its emission spectrum (exitation 440nm; emission 470-700 nm). Albumin, nitro- albumin and glyco-albumin were co-incubated with Aβ40 (Anaspect) and ThT (0.125µg) in a black 96-well plate for 22h. Aβ40 was co-incubated with albumin and modified albumin in a 1:2 molar ratio. The measurements were made with the plate reader spectrophotometer (FLUOstar optima, BMG labtech).

IV. DISCUSSION

NO is a Janus molecule. It works as the major vasorelaxant agent in the systemic circulation as well as the main retrograde neurotransmitter, therefore allowing the process of memory and learning in the brain. These physiological regulatory effects are associated to health and the well-functioning of vessels and brain. But aging and neurodegenerative processes, especially AD, affect dramatically NO's physiology turning it into a strong pathological agent. NO becomes harmful when it is produced in a pro-oxidant environment because it produces peroxynitrite that modifiy irreversibly Tyr residues from proteins and it disrupts the normal function of NO downstream signalling [539-541].

AD affects mainly memory although the damage spreads to other cognitive function as it advances. Despite more than 100 years of research in AD field, its pathological mechanisms are not well understood. Many investigation lines have proposed different mechanisms to be playing a key role in AD onset, but the hypothesis termed "The amyloid cascade" seams the most feasible. This suggests that Aβ aggregation triggers oxidative stress and excitotoxicity due to a maintained activation of the pathway Glu/NMDARc/nNOS [542, 543].

1. Physiology of memory and AD

Memory is a cognitive function to store and recover information. It is produced as a result of a repetitive stimulation of neuronal synaptic connections producing long-lasting changes by LTP or LTD, enhancing or depressing some neuronal nets. The anatomical regions involved in memory formation are mainly the hippocampus

and the cortex. While in the hippocampus recent memory (specially working memory) is stored, cortex takes the storage of long time memories. In AD, these regions are damaged due to the high presence of senile plaques, NFT and neuronal death.

The current Western societies have increasing life expectancies and AD will represent one of the most prevalent diseases in the near future. To discover the pathways involved in this pathology is necessary to understand the physiological mechanisms of memory. Therefore we focused in the study of the glutamatergic signalling, in particular in the signalling via NMDARc. It acts as a coincident detector, that is, it can integrate two stimuli to produce and reinforce the response enhancing the potentiation of one impulse. To be completely activated, NMDARc have to sense the depolarization provoked by AMPARc, release the Mg^{2+}, which is blocking its pore, and binding Glut and Gly. Then, when NMDARc are activated they allow the Ca^{2+} entry inside the cell, triggering different pathways depending on where they are located. When NMDARc is located in the synaptic zone, the intracellular Ca^{2+} activates the CaM, activating directly the nNOS [544, 545]. The nNOS is bound to the cytoplasmatic portion of GluN2B subunit through PSD-95 [322]. Once it is activated it produces NO postsynaptically, which spreads to the presynaptic terminal where it is sensed by the heme group of the GC. The activation of GC produces cGMP [546, 547], causing the Glut release in the synaptic cleft and magnifying the response by its binding to NMDARc. Therefore, a continuous loop of activation is maintained. It is important to highlight the role of NO in this situation, having an active part in the bidirectional

communication between the presynaptic and postsynaptic neuron. In the present work we propose that NO is also contributing in another way to the synaptic function, mainly regulating the expression of different synaptic proteins.

2. Protein synthesis in LTP

To consolidate synapses protein synthesis is needed. Synthesis is started by transcription through the CREB transcription factor that is in turn activated by NMDA signalling. The effects triggered by CREB are long-lasting because it enhances the synthesis of proteins related to dendritic growth and synaptic spines. But gene transcription is a process that requires a lot of time, while protein synthesis is needed quickly in neuronal plasticity especially to initiate and maintain LTP. To solve this time-conflict, several mRNAs are previously transported into dendrites waiting to be translated after synaptic activity [548-550]. In dendrites, there are also the polyribosomes necessary to produce the translation. This process will allow the availability of new proteins in very short time. In fact it has been reported that the mRNA of GluN2A but overall GluN2B is located in neurites [551, 552]. The rapid expression of GluN2B subunits in dendrites could be explained by its quick translation. Once expressed, the GluN2B subunit assemblies with the GluN1 subunit, which is synthesized in excess compared with GluN2 (estimated to be ≈ 10-fold) [553](Huh and Wenthold, 1999; Wenthold et al 1993) and form active receptors.

3. NO regulates mRNA translation through HRI activation

Following the theory of quick availability of GluN2B subunit by translation in dendrites, we tested the system of the 5'UTR repression. GluN2B has a 5'UTR with 179 bp, which contains three uAUG. When we tested the repression activity of the 5'UTR by a reporter assay, we found that the 5'UTR is able to repress 80% of the luciferase (reporter gen) expression. However, this repression is reverted in a 60% with the triple mutant of the three uAUG. This supports the idea that the repression of the GluN2B translation is mainly due to its uATG. Interestingly, we found that the treatments with NO, as well as with NMDA or Sal, an inhibitor of PP1 which dephosphorylates eIF2α, were able to de-repress the luciferase expression. This was indicating a key role of NO in the regulation of eIF2α phosphorylation, and the only possible candidate had to be via the HRI kinase.

HRI belongs to the group of eIF2α kinases, cellular stress sensors that after being activated by any source of cellular stress phosphorylate eIF2α to arrest global protein synthesis. This is the biological cost of using a stress activated effector such as eIF2α for the fine regulation of translation initiation in the synapse. The role of eIF2α in memory has been previously related with mRNA translation during synaptic plasticity through its phosphorylation by the activation of GCN2 [554-557]. The activation of eIF2α kinases produces the eIF2α-phosphorylation and the inhibition of the general mRNA translation except those ones having a 5'UTR with several AUG.

In the glutamatergic signalling GluN2B would be translated in the presence of eIF2α phosphorylated, because the scanning process would be more efficient, reaching the main AUG. We found that this effect is dependent on HRI activation by NO (**Fig.11**). To demonstrate this we have used cortical neuronal cultures and synaptosomes. The laters are highly important for our hypothesis since synaptosomes have all the translational machinery but not the transcriptional one. Consequently we found that the treatment with NO increases the levels of p-eIF2α correlating with an increased expression of GluN2B. The specificity of this pathway was demonstrated by using a HRI inhibitor that prevented these increases as well as the inhibitor of nNOS, 7-NI. NMDARc stimulation by its pharmacological ligand NMDA also triggered the production of NO by the activation of nNOS through Ca^{2+}/CaM. Hence, NMDA also induces GluN2B expression supporting the idea that NO is the main responsible of this mechanism. Other approach to demonstrate that GluN2B expression depends on eIF2α phosphorylation was performed by using Sal. As expected, Sal also increased GluN2B expression. We also demonstrated that this increase correlated with a higher entry of Ca^{2+} due to GluN2BRc. We consider that understanding the physiology of this mechanism is crucial to comprehend the deleterious misregulation in AD pathology.

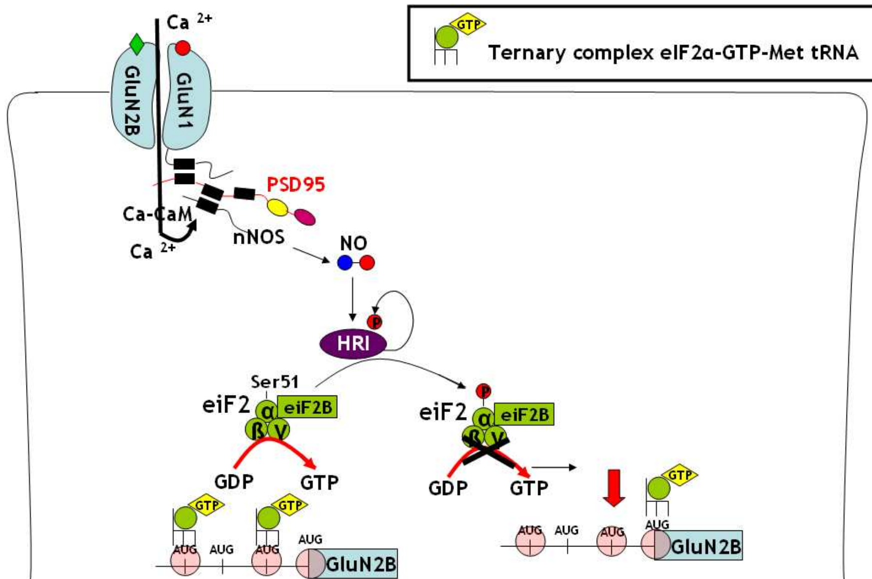


Figure 11. ***GluN2B translation mediated by HRI activation and eIF2α phosphorylation***. *Postsynaptic ending showing how NO activates HRI and stimulates eIF2α phosphorylation. The phosphorylation enhances the GluN2B translation.*

4. The misregulation of NMDARc

The regulation of NMDARc subunits is crucial since an overexpression or a decrease can produce LTP impairment. It has been also reported that an overstimulation of NMDARc produces excitotoxicity and neuronal death [558], processes in where GluN2BRc would be playing a key function. The GluN2BRc correlates with pro-death pathways due to its predominant location in extrasynaptic places and its ability to allow greater Ca^{2+} influx [559]. Thus, an increase of extrasynaptic glutamatergic activity instead of the synaptic one would be happening in AD. In fact, the effect of memantine to slows the disease progression in subjects with

moderate to severe AD [331, 560, 561], is thought to be by the blockage of NMDARc opening due to prolonged exposure to extrasynaptic Glut without blocking the physiological activation [562].
On the other hand there are studies reporting a GluN2BRc decrease in AD by endocytosis [352, 353] or even a lower expression in brains[563]. These findings fit with a decrease of NO bioavailability. In AD most of the synthesised NO would be scavenged by $O_2^{\cdot -}$. Consistently we found a significant increase of GluN2B nitrotyrosination in AD brains supporting a high nitro-oxidative stress. GluN2B subunit contains 55 Tyr able to be modified by $ONOO^-$. Some Tyr have key roles as those in the ligand binding domain [564], and Tyr231 [565] and Tyr282 [566] placed in the amino terminal domain, whose nitrotyrosination can affect the deactivation or the channel's open probability. Further work is needed to clarify the consequences of these modifications in the activity of the channel. In any case, it would fit with a less amount of GluN2BRc expression upregulated by NO. Under these stressful conditions the phosphorylation of eIF2α instead of being mediated by HRI would be carried out by PERK, PKR or GNC2, increasing the GluN2B subunit by other pathological and unregulated pathways. Then the reported decrease of GluN2BRc in AD brains would be related to massive Ca^{2+} entries and death of the glutamatergic neurons [324, 567].

5. Aging and oxidative stress

Aging is a progressive, endogenous, irreversible and deleterious process characterized by a gradual functional decline of all the body systems. Concretely, CNS aging produces intellectual and memory

deterioration by the structural neuronal changes and the loss of neurons in some brain areas.

Among the many theories about the molecular causes of aging, the free radicals theory due to mitochondrial failings, proposed by Harman [568], is the most accepted. Supporting this idea it has been reported increased levels of oxidizing species with age. The increase of ROS by mitochondria metabolism can have many deleterious effects and albeit the antioxidant system tries to counteract oxidative damage, free radicals can accumulate during the life cycle, overcoming the defense mechanism. In fact there is a correlation between mitochondrial ROS production and longevity, observed at least for a few species [569, 570].

Oxidative stress could explain the modifications during brain's aging, which would happen more pronouncedly in neurodegenerative diseases like AD [571].

6. AD and oxidative, nitrative and glycative stress

Since the brain is the organ with the major consume of oxygen (20% of the total glucose) it is subjected to a high oxidative stress. The ROS increase during aging can be the result of an unbalance between the production and its destruction. In the case of AD patients, the oxidative stress [572, 573] is magnified by the Aβ extracellular deposition. Aβ produces ROS through the release of H_2O_2 by metal ion reduction [137, 574, 575]. Oxidative stress is considered to be the cause of amyloid toxicity. In this scenario, the NO synthesized after NMDARc activation is mostly scavenged by the $O_2^{\cdot -}$ producing $ONOO^-$. It decreases the bioavailability of NO in

detriment of the reactive and toxic specie $ONOO^-$. $ONOO^-$ nitrates tyrosines changing the protein's normal properties by decreasing their activity. Several proteins are described to be nitrotyrosinated in AD [576, 577] like the triosephosphate isomerase (TPI) [578]. The effects of TPI nitrotyrosination has been widely studied by our group. Mainly, it generates a bigger aperture of its catalytic center, producing a major production of the metabolite MG. MG acts as a highly reactive species, which glycates proteins, modifying and altering their structure and function. This goes in agreement with studies reporting an increase of glycative and nitrative stress in AD[579-582].

7. Albumin as a scavenger of oxidative damage

Albumin, the most abundant protein in blood, is considered to be a protective molecule since it has an antioxidant function by quenching metal ions, ROS and NO [453, 583] through its free Cys [487, 584-587] and its DAHK domain in it N-terminal region [588, 589]. Likewise, the bovine homolog protein, BSA, exhibits SOD-like activity by inhibiting $O_2^{\cdot-}$ formation and reducing lipid peroxidation [590], leading to a decreased oxidative stress in the cell.

8. Albumin modifications in nitro-oxidative conditions

Albumin can be modified by nitrative and glycative stress during AD. Some works have previously demonstrated that albumin can be altered by glycation and nitration and that both process change its structure [467, 532, 591]. These modifications would be impairing its

function, avoiding its scavenging properties or affecting the binding of molecules such as Aβ.

In our studies we have obtained that albumin is significantly nitrated and glycated in brain parenchyma and in blood from AD patients compared to healthy controls. The relevance of these findings was studied by the nitration and glycation of albumin *in vitro*. We performed assays with $ONOO^-$ and MG demonstrating that both compounds produce the nitration and the glycation of albumin, inducing its aggregation. This should be due to the formation of dityrosine bridges between the nitrotyrosines in the case of ONOO- treatment, or the crosslinking between glycated residues in the case of MG treatment. Although it aggregated faster, nitroalbumin was not able to produce the big aggregates as glycoalbumin does. Actually the biggest aggregates formed by glycoalbumin were not able to pass through the acrylamide pore (8% of acrylamide) of the gel in the WB assay. The structure of the albumin aggregates was studied by electron microscopy and the nitroalbumin showed different and denser aggregates regarding glycoalbumin or the normal albumin. This is due to the differential mechanism involved in its aggregation (dityrosine bridges *vs* cross-linking).

One of the pathophysiological effects of the albumin aggregation was a decrease in the osmotic pressure by the binding of ions, resulting in a decrease of 20% in the osmolarity of the solution. The effect on the oncotic pressure is assumed since the number of albumin monomers is decreased when albumin aggregates. Physiologically, this effect can have serious consequences

producing liquid extravasations through small brain vessels [518, 592, 593] avoiding the normal exchange through the BBB.

Likewise, these aggregates formed by nitrotyrosination or glycation would make albumin more resistant to be degraded. In fact it has been reported that crosslinked proteins are less susceptible to proteolysis having harmful implications in age and disease-related impairment of cellular function [594, 595]. This critical point was assayed with hepatoma cells since liver is responsible of albumin degradation and production. We obtained a less uptake by these hepatic cells when albumin was nitrotyrosinated or glycated. This would mean a higher systemic circulating time of the modified and non-functional albumin.

On the other hand, nitrated and glycated albumin could have harmful effects on the cells of the vessels and brain parenchyma. To assess this issue, we tested how the modified albumin affects endothelium, vascular smooth muscle cells, glial cells and neurons. We obtained that modified albumin was toxic for all cells but endothelium. Interestingly the proportional amount of pure ONOO- or MG, which was used to nitrate and glycate albumin, was highly toxic for all cells. This suggests that nitration and glycation of albumin can be a first line of defense to scavenge these species to avoid their harmful effects.

In AD, albumin is playing a key role by binding Aβ that has to be degraded in the liver. Albumin is responsible for the 95% of Aβ transport in blood [596-599] regulating the amount of soluble Aβ in the circulation. The availability of soluble Aβ in blood can determine the Aβ entrance in brain through RAGE, being important in the

brain amyloid deposition and AD progression. Therefore we studied albumin binding ability to soluble and aggregated forms of $A\beta_{1-40}$, the most abundant form of Aβ. Nitro- and glycoalbumin bound more soluble $A\beta_{1-40}$ than unmodified albumin. Besides, glycoalbumin also bound more aggregated $A\beta_{1-40}$ than the unmodified albumin. The modified albumin bound to $A\beta_{1-40}$ would remain more time in the circulation due to its difficulty to be degraded by the liver. This will affect its Aβ binding ability because there will be no free albumin to bind more Aβ. Consequently, the flux of Aβ from brain to vessels will be impaired, increasing the Aβ concentration inside the brain.
The pathophysiological importance of these results is highlighted by our findings demonstrating that in brain as in plasma samples from AD patients there are more nitro- and glycoalbumin. This postulates albumin not only as a marker of the AD progression, but also an active part of the deleterious mechanisms happening during the development of AD.

9. Final considerations

The results obtained with this thesis pretend to put a little bit of light on the effects of NO in the synaptic activity and in the mechanisms involved in AD onset and progression. The study of the dual role of NO highlights the need of a tight regulation of the biological process. NO effects are an example of how an unbalanced system may increase the molecule concentration resulting in a pathological state. An abnormal environment produces a tendency to lose its physiological function shifting to the

pathological effects. Initially, this tendency would act as a compensatory mechanism when physiological mechanism are not working properly, but if the same condition persists it can activate permanently the pathways that mediates protein and cell damage. Consequently, it is important to understand the biochemical mechanisms of NO signalling to distinguish the fine line that separates health from disease. Only in that way we will be able to take in account all the factors contributing to the disease's cause and see the whole picture of AD.

V. CONCLUSIONS

1. NO activates HRI kinase, phosphorylating the eIF2α factor and producing the GluN2B subunit translation.

2. The three uAUG codons in the 5’UTR repress the physiological translation of GluN2B.

3. The increase in GluN2B translation produces functional channels.

4. The Glut-NO-HRI-GluN2B pathway would be playing an important role in synaptic processes like memory and learning due to its synaptic location.

5. GluN2B location in extrasynapses areas can modulate excitotoxicity in neurons.

6. GluN2B subunit is more nitrotyrosinated in AD patients compared to controls due to the higher nitrooxidative stress environment.

7. Albumin is more glycated and nitrotyrosinated in brain and blood samples from AD patients compared to controls.

8. The nitrotyrosination and glycation of albumin changes its properties promoting its aggregation, decreasing its ability to buffer the osmolarity and making it toxic.

9. The glycated and nitrotyrosinated albumin binds more Aβ in the soluble form and glycated albumin also binds more aggregated Aβ than the unmodified one.

10. The modified albumin remains more time in blood bound to Aβ, worsening the progression of the disease.

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VII. ANNEX

ANNEX 1

Amyloid β Peptide Fibrils Induce Nitro-Oxidative Stress in Neuronal Cells.

Gerard Ill-Raga, Eva Ramos-Fernández, Francesc X. Guix, Marta Tajes, Mònica Bosch-Morató,Ernest Palomer, Juan Godoy, Sebastián Belmar, Waldo Cerpa, JamesW. Simpkins,Nibaldo C. Inestrosaand Francisco J. Muñoz,

This paper was presented as part of the results of Gerard Ill-Raga's PhD Thesis entitled "Study of the physiopathological role of nitric oxide and nitrative stress in brain: translational effects on the cleavage of the amyloid precursor protein in Alzheimer's disease and post-translational effects of fibrinogen in brain ischemia"

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ANNEX 2

PKR and PP1C Polymorphisms in Alzheimer's Disease Risk

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ANNEX 3

Activation of PKR Causes Amyloid ß-Peptide Accumulation via De-Repression of BACE1 Expression

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This paper was presented as part of the results of Gerard Ill-Raga's PhD Thesis entitled "Study of the physiopathological role of nitric oxide and nitrative stress in brain: translational effects on the cleavage of the amyloid precursor protein in Alzheimer's disease and post-translational effects of fibrinogen in brain ischemia"

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ANNEX 4

Modification of γ-secretase by nitrosative stress links neuronal ageing to sporadic Alzheimer's disease

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ANNEX 5

The pathophysiology of triose phosphate isomerase dysfunction in Alzheimer's Disease

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The pathophysiology of triose phosphate isomerase dysfunction in Alzheimer's disease

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Running title: TPI and Alzheimer disease

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Abstract (190/250)

Alzheimer's disease (AD), the most prevalent neurodegenerative disease worldwide, has two main hallmarks: extracellular deposits of amyloid ß-peptide (Aß) and intracellular neurofibrillary tangles composed by tau protein. Most AD cases are sporadic and are not dependent on known genetic causes; aging is the major risk factor for AD. Therefore, the oxidative stress has been proposed to initiate the uncontrolled increase in Aß production and also to mediate the Aß's deleterious effects on brain cells, especially on neurons from the cortex and hippocampus. The production of free radicals in the presence of nitric oxide (NO) yields to the peroxynitrite generation, a very reactive agent that nitrotyrosinates the proteins irreversibly. The nitrotyrosination produces a loss of protein physiological functions, contributing to accelerate AD progression. One of the most nitrotyrosinated proteins in AD is the enzyme triosephosphate isomerase (TPI) that isomerises trioses, regulating glucose consumption by both phosphate pentose and glycolytic pathways and thereby pyruvate production. Hence, any disturbance in the glucose supply could affect the proper brain function, considering that the brain has a high rate of glucose consumption. Besides this directly affecting to the energetic metabolism of the neurons, TPI modifications, such as mutation or nitrotyrosination, increase methylglyoxal production, a toxic precursor of advanced glycated end-products (AGEs) and responsible for protein glycation. Moreover, nitro-TPI aggregates interact with tau protein inducing the intraneuronal aggregation of tau. Here we review the relationship between modified TPI and AD, highlighting the relevance of this protein in AD pathology and the consequences of protein nitro-oxidative modifications.

Key words: Alzheimer's disease; triosephosphate isomerase; oxidative stress; nitrotyrosination; methylglyoxal

Introduction

Aging is characterized by a lack in redox homeostasis. Consequently, there is an increase in nitro-oxidative stress, which plays a key role in the onset and progression of neurodegenerative processes (Di Monte et al., 1992; Omar and Pappolla, 1993; Halliwell, 1992; Miranda et al., 2000; Guix et al., 2005). Nitro-oxidative stress is directly related to mitochondrial dysfunction (Schon, 1997), calcium deregulation (Mattson et al., 1992) and protein aggregation (Guix et al., 2009; Kummer et al., 2011), which induce neuronal death (Butterfield and Boyd-Kimball, 2004; Pratico et al, 2001; Ill-Raga et al., 2010). All these features are found in Alzheimer's disease (AD).

Alzheimer's disease

AD hallmarks

AD is the most common form of dementia in the elderly, accounting for 60–70% of all cases and affecting 10% of individuals older than 65, and nearly 50% of those older than 85 (Malenka and Malinow, 2011 ; Imbimbo et al., 2005). It courses with progressive deterioration of memory, behaviour and cognition because of major neuronal damages in the hippocampus and neocortex. Memory decline initially manifests as a loss of episodic memory, impeding recollection of recent events, including autobiographical activities. The progression of the disease causes a dramatic decline in cognitive abilities (Ball et al., 1985). The specific therapies for AD address the cholinergic deficit and the overstimulation of the glutamatergic NMDA receptors, but they fail to avoid the progression of the disease.

AD can be classified into two types depending on the age of the disease onset. The early onset AD is known as Familial AD (FAD) due to the mutations in proteins such as amyloid precursor protein (APP) or presenilins (PS) (Tanzi el al., 1992; Levy-Lehad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). FAD appears before the patients are 65 years old, being less common (less than 5% of the total AD cases) than the late onset one, but it progresses very quickly. Late onset AD is the most common AD and it has been related to some polymorphisms, especially with ApoE4 (Saunders et al., 1993; reviewed in Wasco and Tanzi., 1995).

Both types of AD are characterized by neuronal death associated to extracellular amyloid ß-peptide (Aß) deposits and intracellular neurofibrillary tangles (NFT), composed of tau protein. Aß is a product of the cleavage of APP by the sequential action of ß-aspartyl secretase (BACE1) and γ-secretase (PS complex). Aß is able to aggregate in ß-sheet, forming primary structures called oligomers (dimers, trimers and tetramers) that can assemble to form

protofibrils (PF) as intermediate structures between aggregates and mature fibrils. Oligomers are considered the most toxic Aß forms (Wang et al., 2002; Kelly and Ferreira, 2006; Shankar et al., 2007), remaining in the proximity of the neuronal membranes where they produce their harmful effects. Currently, the mature fibrils aggregation forming senile plaques and brain vascular deposits of amyloid are considered a mechanism to avoid the high oligomer neurotoxicity.

On the other hand, NFT are composed of tau aggregates, a microtubule associated protein which, detached from microtubules, aggregates to form the paired helicoidal filaments (Morishima-Kawashima et al., 1995; Braak et al., 1994; Bramblett et al., 1993; Yoshida and Ihara, 1993). Tau is hyperphosphorylated when forming NFT, which has produced a search for different kinases, such as glycogen kinase 3-beta (GSK-3ß), to be responsible for NFT formation (Illenberger et al., 1998; Moreno et al., 1996). The relevance of NFT in AD is supported by the correlationship between their presence and the dementia level, a fact that is impossible to establish with senile plaques, probably due to the major effect of oligomers, which are histochemically "invisible".

Aß and nitro-oxidative stress

There is much evidence relating AD pathology with nitro-oxidative stress. Aß aggregation into ß-sheet induces the production of free radicals due to the reduction of transition metals (Huang et al., 1999; Varadarajan et al., 1999). Misfolded Aß is capable of binding Cu (II) and Fe (III) and reduce these transitional metals to Cu(I) and Fe(II), producing hidroxyl radicals and H_2O_2, which causes cytotoxicity (Huang et al., 1999; Cuajungco et al., 2000; Muñoz et al., 2002) by inducing lipid peroxidation, protein oxidation, nitrotyrosination and glycation, and DNA oxidation (Miranda et al., 2000) (Fig.1).

The damage in membrane transporters and ion channels leads to an increase in intracellular calcium levels (Mattson et al., 1992; reviewed in Yu et al., 2009). It produces the synthesis of nitric oxide (NO) by the neuronal NO synthase (nNOS), since it is a Ca^{2+}-calmodulin-dependent enzyme (Guix et al., 2005). NO has an unpaired electron in the last orbital acting as a free radical (Stamler et al., 1992). Hence, NO can react with other molecules such as superoxide anion ($O_2^{\cdot-}$), forming peroxynitrite anion (ONOO-) (Beckman et al., 1990), a short lived molecule but highly reactive. Peroxynitrite nitrotyrosinates proteins, a process which consists of the addition of a nitro group (NO_2) to tyrosine residue (Ischiropoulus et al., 1992). Nitrotyrosination is highly spread in AD brains (Hensley et al., 1998).

In our lab we have demonstrated that one of the proteins most nitrotyrosinated due to Aß action is triosephosphate isomerase (TPI) (Coma et al., 2005; Guix et al., 2009), a key enzyme in the cell metabolism that controls glycolytic flow and energy production (Richard et al., 1993).

Triosephosphate isomerase

TPI cellular function

TPI is an enzyme that catalyses the interconversion of D-glyceraldehyde-3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP) from both the glycolytic and phosphate pentose pathways, the latter being the most active glucose metabolic pathway in neurons (Bolaños et al., 2010) in order to increase antioxidant protection by producing GSH.

The rate of the catalysis is diffusion-limited, and the equilibrium favours the formation of DHAP by 1:20 (Olah et al., 2002). TPI is essential for the efficient energy production of glycolysis; therefore it is critical for the functional activity of the cells (Fig.2). Interestingly TPI has been proposed to be affected in aging, altering energy metabolism (Hipkiss, 2011).

GAP is diverted to pyruvate producing four adenosine triphosphate (ATP) molecules. In brain cells DHAP is not a dead-end product and it can be directed to lipid synthesis (Kusaka et al., 2007). In fact, the glycolytic pathway is interconnected with the lipid metabolism, the pentose phosphate pathway, and the gluconeogenesis pathway via GAP and/or DHAP. The metabolic flow through these pathways will be affected in the case of deficiencies in TPI activity (Orosz et al., 2009; Richard, 2008).

The deficiency of this enzyme is characterized by haemolytic anaemia. Interestingly, it is the only glycolytic enzyme defect that is associated with neurodegeneration (Olah et al., 2005). The deficiencies in TPI activity do not just affect cells by the reduction in ATP and pyruvate supply, but also by the formation of methylglyoxal (MG) (Ahmed et al., 2003). This is a toxic triose formed at a very low rate as a side-product of TPI, but its production increases when TPI is damaged (Guix et al., 2009; Fig. 3). MG modifies proteins by the glycation of different aminoacids.

TPI structure

TPI is a stable homodimer of two 27 kDa subunits. Although every single monomer has the residues for the catalytic activity, the dimerisation apparently rigidifies each of the two separate active sites, providing full catalytic power and being active just in its dimeric form (Mainfroid et al., 1996; Wierenga et al., 2010). TPI dimer is the most common quaternary structure, but in thermophylic organisms TPI is known to occur as tetramers (Maes et al., 1999). At present, there are at least 118 crystal structures of TPIs in the PDB (RCSB Protein Data Bank). Structural studies have shown that the active site is at the dimer interface, with all catalytic residues (Asn11, Lys13, His95 and Gly167) for a particular active site coming from the same subunit. Several water molecules are an integral part of the dimer interface, and six of

them are highly conserved (Thakur et al., 2009). TPI has four tyrosines. The first two, Y47 and Y67, stay at the interface of the dimer in opposite orientations, while the remaining two, Y164 and Y208, interact directly and locate very close to the catalytic site (Guix et al., 2009; Fig.4).

The spatial structure of TPI is a $(\beta/\alpha)_8$ barrel fold, also known as a "TIM-barrel". This fold consists of a regular eightfold repeating pattern of ß-strands and α-helices. The ß-strands form the inner set of eight parallel ß-strands, covered on the outside by the subsequent α-helices (Nagano et al., 2002). The α-helices and ß-sheets are linked by loop regions. Three loops of the N-terminal half of the molecule are involved in the intersubunit interactions, another three participate in the active site. Specifically, loop-1 has the residues Asn11 and Lys13; loop-4 has His95 and loop-6 has Gly167 (Orosz et al., 2009; Wierenga et al., 2010).

Loop-6 is very flexible and plays an important role in substrate binding and catalysis. In the unliganded conformation, loop-6 interacts with loop-5, whereas in the closed/liganded conformation it interacts with loop-7 (Wierenga et al., 2010). Any variation in loop-6 could affect the eficiency of the enzyme. A TPI variant, in which four residues of that loop have been removed, increases the synthesis of the toxic MG (Pompliano et al., 1990). Similar results were obtained by our group when TPI was mutated at Tyr164 and Tyr208 by Phe or by inducing the nitrotyrosination of the enzyme (Guix et al., 2009).

TPI deficiencies

TPI is coded by one gene located at chromosome 12p13 in the human genome. Its amino acid sequence is highly conserved among all known TPI proteins (Schneider et al., 2000). There are TPI deficiencies due to autosomal recessive multisystem genetic disorder, characterized by decreased enzyme activity, which is accompanied by an increase of DHAP. This deficiency is manifested clinically, like many glycolytic enzymopathies, as chronic hemolytic anemia, although this disorder is unique among the glycolytic enzyme defects associated with progressive neurological dysfunction and, frequently, childhood death (Orosz et al., 2006; Orosz et al., 2009, Olah et al., 2005). The pathogenesis of this disease is not well understood, and no effective therapy is available. However, there are experiments showing the normalization of DHAP levels in TPI-deficient cells treated with the active form of the enzyme (Ationu et al., 1999).

Patients with various inherited mutations have been identified. The most abundant missense mutation in humans occurs at codon 104 in the TPI gene (Glu104Asp mutant). This mutation is not only the most common, but also causes the most severe symptoms (Schneider et al., 2000; Orosz et al., 2009).

There are several theories to explain the low activity in TPI deficient cells, but most of them have in common the instability of the enzyme. Any mutation or modification in the

subunit interface results in loss of activity, due to the dissociation of the active dimers into inactive monomers (Ationu et al., 1999), or aberrant dimerization (Orosz et al., 2009), and these changes could play a crucial role in the etiology of the illness. The heteroassociations with different cellular structures, such as microtubules in neurons, result in alterations in the catalytic and regulatory properties of the enzymes (Ovadi et al., 2004). Finally, another possible theory is the fact that a perturbation of the conserved network of buried water molecules that bridge the two subunits appears to be essential to maintain the stability of TPI dimers (Rodríguez-Almazán et al., 2008).

Bioinformatic analysis, based on the 3D structure of the wild-type enzyme, was used by Schneider *et al.* (2000) to explain the structural and catalytic properties of the mutant enzymes observed in the patient's hemolysates. They mapped the amino acid residues, as well as the first and second degree contacts of all the residues comprising each of three functional domains of TPI substrate binding, flexible loop and dimer interface domains (Schneider et al., 2000).

Susan Hollán (1993) reported a very interesting case in a Hungarian family with two germ-line identical but phenotypically different heterozygote brothers who inherited two independent mutations in TPI enzyme, Phe240Leu and Glu145stop codon (Hollán et al., 1993). The activity of TPI was dramatically reduced in both brothers, resulting in 40-60-fold higher DHAP concentration in their erythrocytes as compared with normal controls (Valentin et al., 2000; Eber et al., 1991). However, only the younger sibling (affected brother) manifests neurological disorders. This fact may provide key information about the etiology of neurodegenerative symptoms associated with TPI deficiency. Some of the features that are only present on the neurological affected brother are:

i) A decrease in membrane plasmalogen and changes of membrane reactivity and fluidity, enzyme activities, signal transduction and sensitivity towards oxidative stress.

ii) Imbalance of the prooxidant/antioxidant homeostasis, highly related with neurodegeneration

iii) An increase in the expression of endothelial NOS and a decrease in POP (prolyl-oligopeptidase). High NO production is responsible for the broad protein nitrotyrosination (Coma et al., 2005) while POP plays a key role in neurotransmission and intracellular protein degradation, and its reduction contributes to the development of neurodegeneration (Ahmed et al., 2003; Orosz et al., 2006).

DHAP increase and its consequences

The most important biochemical feature of TPI deficiency seems to be the dramatic increase in the cellular concentration of DHAP (20-60 fold) overall in erythrocytes. DHAP is involved in lipid metabolism, and its accumulation provokes a disturbance in the lipid balance.

The levels of plasmalogen, an ether lipid, are reduced in TPI deficiency, and as a consequence, the protection against oxidative stress related to this lipid is impaired.

On the other hand DHAP is decomposed by non-enzymatical reaction to MG, a highly reactive glycating agent which is responsible for protein glycation and a precursor of advanced glycation end-products (AGEs). MG is toxic to neurons and may contribute to AD progression (Kikuchi et al., 1999; Orosz et al., 2006). Under oxidative stress conditions glyoxalases cannot efficiently detoxify MG, which may underlie the associated neurodegeneration (Ahmed et al., 2003; Fig. 3).

TPI and Alzheimer's disease

The nitration of tyrosines occurs in young and aged individuals, but it is increased in the latter. Certain levels of nitrotyrosination can be managed by the organism eliminating the damaged proteins, but when the process is accelerated it represents a pathological event that is associated with neurodegenerative diseases, in particular with AD (Smith et al., 1997). Specifically, $O_2^{\cdot-}$superoxide anion, produced by Aß cell damage, and NO, whose production is altered in AD, react to form the highly reactive peroxynitrite anion, which generates cytotoxic species that oxidize and nitrate proteins (Castegna et al., 2003; Guix et al., 2005).

Glucose is the primary source of energy for the brain, and the interruption of glycolysis causes brain dysfunction and memory loss, favoring neurodegeneration. In fact, inefficient glucose metabolism is characteristic in AD (Hoyer et al., 1996). A plausible explanation is that TPI is one of the proteins most nitrotyrosinated in AD (Coma et al., 2005; Butterfield et al, 2006b; 2007) and when nitrotyrosinated it decreases TPI isomerase activity, reducing the glycolytic flow, and increasing MG production (Guix et al., 2009). The relevance of nitrotyrosination in this effect was shown when TPI was mutated at Tyr164 and Tyr208 by Phe, mimicking TPI nitrotyrosination, and producing similar results (Guix et al., 2009).

Since a lower amount of pyruvate would be available for neurons, mitochondrial activity can be decreased. There are no works addressing this scenario, but a lower acetyl-CoA bioavailability can be expected, and one of the consequences could be related with a decreased production of acetylcholine (ACh), contributing to the characteristic cholinergic deficit in AD (Schliebs and Arendt, 2011).

Moreover, TPI nitrotyrosination as well as TPI mutations induce the aggregation of the enzyme, forming several ß-strands (Rice et al., 1990), a process likely favored by its homology in the sequence with the Aß peptide (Contreras et al., 1999). The presence of intracellular nitro-TPI aggregates into ß-sheets was demonstrated in immunoprecipitated samples from AD cortex (Guix et al., 2009). Interestingly, TPI from subjects with heterozygote variants of mutated enzyme, bound more strongly to microtubules than TPI from normal controls. The mutation in the enzyme could lead to aberrant protein-protein interaction (Ovadi et al., 2004), affecting the

trafficking machinery of the cell (Bonnet et al., 2004). In the same direction, nitrotyrosinated TPI aggregates are able to bind tau protein, a microtubule associated protein, inducing a conformational change in tau that precipitates paired helical filament formation, the other hallmark of AD (Guix et al., 2009; Fig. 5). It would link the effects of Aß oligomers and fibrils with the characteristic intraneuronal tau aggregation and neurodegeneration (Fig. 6).

Conclusions

The nitrotyrosination of the enzyme TPI by Aß aggregates seems to be critical in AD neurodegeneration. Nitro-TPI decreases G3P bioavailability that will affect all cellular functions. Moreover, it produces toxic MG, damaging proteins irreversibly. Besides this metabolic and toxic effect, nitro-TPI can induce the aggregation of tau protein, disassembling the neuronal cytoskeleton and avoiding normal intracellular trafficking and the intercommunication of the neurons.

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Figure Legends

Fig. 1 Aß induces TPI nitrotyrosination. Aß fibrils and oligomers produce free radicals, which damage mitochondria. Consequently, intracellular calcium levels raise and activate the enzyme nNOS. Therefore, NO and superoxide anion react to form peroxynitrite that nitrotyrosinates TPI. In the inset it is shown a western blot of human neuroblastoma cells treated with Aß fibrils and untreated control cells (C). TPI was immunoprecipitated and the western blot was revealed with an antibody anti-nitrotyrosine.

Fig. 2 TPI nitrotyrosination decreases pyruvate production. Neurons metabolize glucose mainly by the pentose phosphate cycle and at a lower rate by glycolysis. Both pathways produce G3P. When TPI is nitrotyrosinated there is a decrease in its isomerase activity and DHAP increases. Pyruvate supply is low and it produces a fall in the mitochondrial membrane potential and acetyl-CoA, the precursor of acetylcholine (ACh), which is a neurotransmitter depleted in AD.

Fig. 3 Protein glycation by TPI nitrotyrosination. (A) TPI induces MG production from DHAP. MG can be metabolized to lactate by glyoxalase system under physiological conditions. When MG production is triggered due to TPI nitrotyrosination, it glycates proteins, damaging them. **(B and C)** The right panels show slides obtained from the hippocampus of a double transgenic mice overexpressing human APP and PS1. Immunofluorescence images were obtained by incubating with anti-human Aß **(B)** and anti-glycated aminoacid antibodies **(C)**. High glycation is observed in the hippocampus of this AD model animal.

Fig. 4 Tyrosines of TPI. TPI has 4 tyrosines. **(A)** Tyrosines 164 and 208, through hydrogen bonding, regulate the hinge movement of loop 6 (residues from 168-178) over the catalytic site formed by Glu165, His95 and Lys13. **(B)** Tyrosine 47 (left) and tyrosine 67 (right panel) are located at the interface of the dimer, probably contributing to its stability and therefore to the activity of the enzyme. The images were obtained by the software Rasmol (www.rasmol.org) from the PDB file 2JK2 containing the structure of human TPI.

Fig. 5 TPI nitrotyrosination induces its aggregation. (A) Immunohistochemical analysis of a cortex sample from an AD patient showing aggregated TPI inside the neurons. The image was obtained using an anti-TPI antibody and peroxidase staining. **(B)** Nitro-TPI aggregates as shown in transmission electron microscopy images obtained with untreated TPI (right) and TPI treated (left) with 50 mM peroxynitrite donor (SIN-1) *in vitro*. **(C)** Nitro-TPI induces tau aggregation as shown in the dot blots performed with samples incubated from 0 up to 72 hours at 1:1 (w/w) ratio. Samples were centrifuged and pellets were washed and sonicated. A representative dot blot is shown after incubation with an anti-tau monoclonal antibody. In the supernatants (right) there is a decrease of free tau when incubated with nitro-TPI from 24 up to 72 h. It corresponds with the results obtained in the pellets (left), where there is an increase in high molecular tau aggregates from 24 up to 72 h.

Fig. 6 Effects of TPI nitrotyrosination due to Aß toxicity. This scheme summarizes all the proposed effects of TPI nitrotyrosination in AD linking Aß with neurodegeneration.

Figure 1

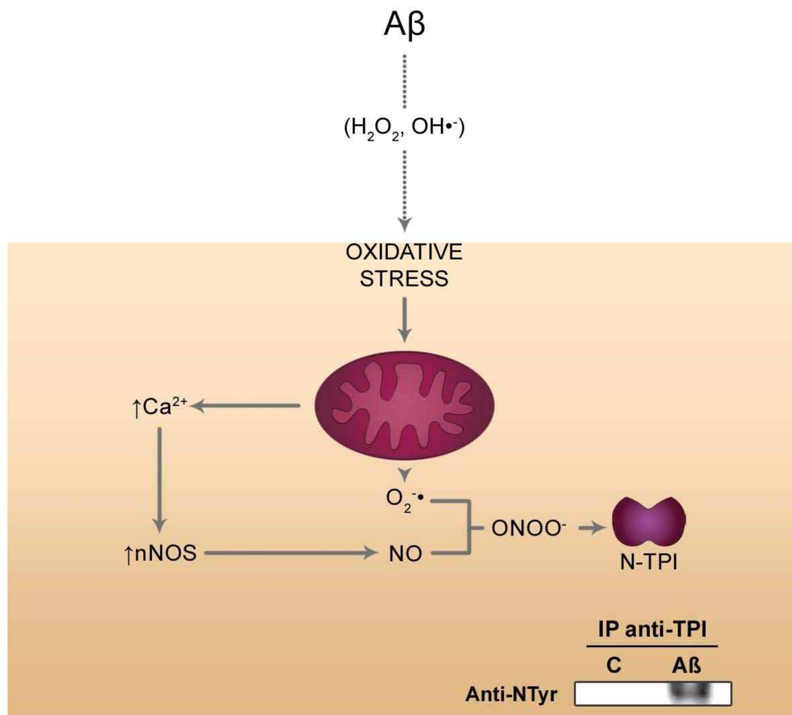

Figure 2

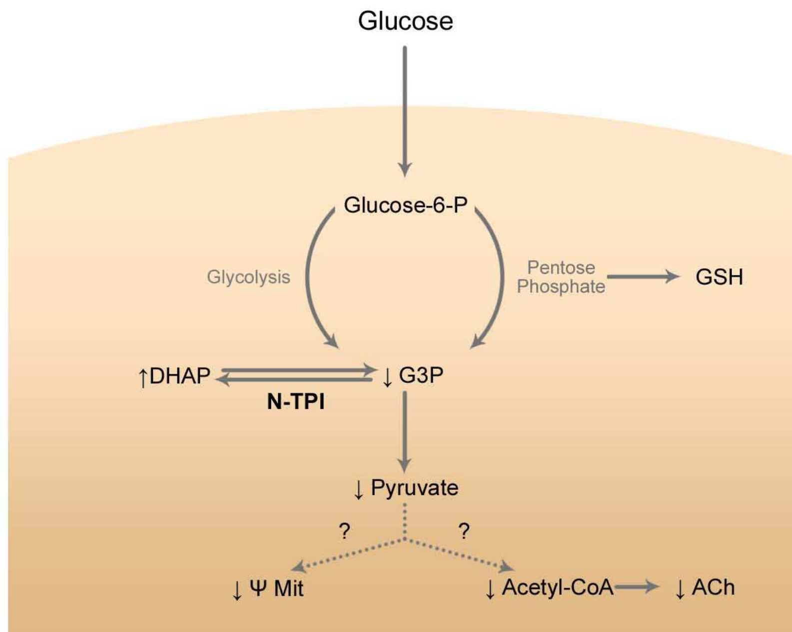

Figure 3

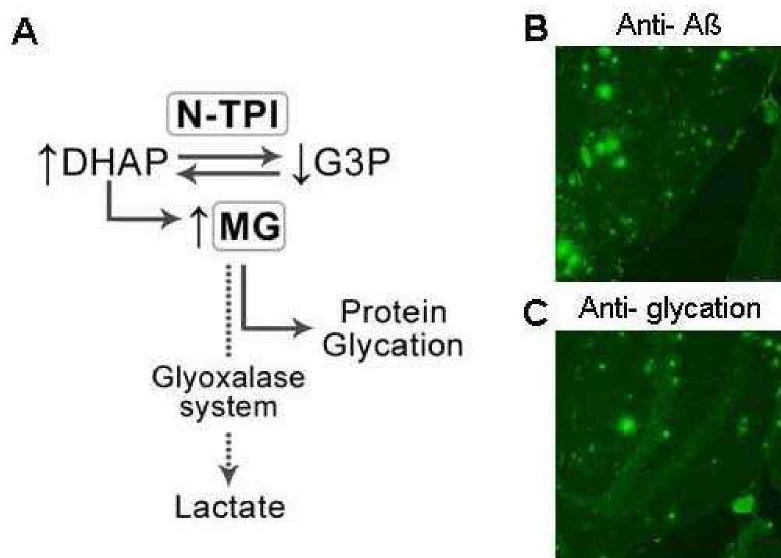

Figure 4

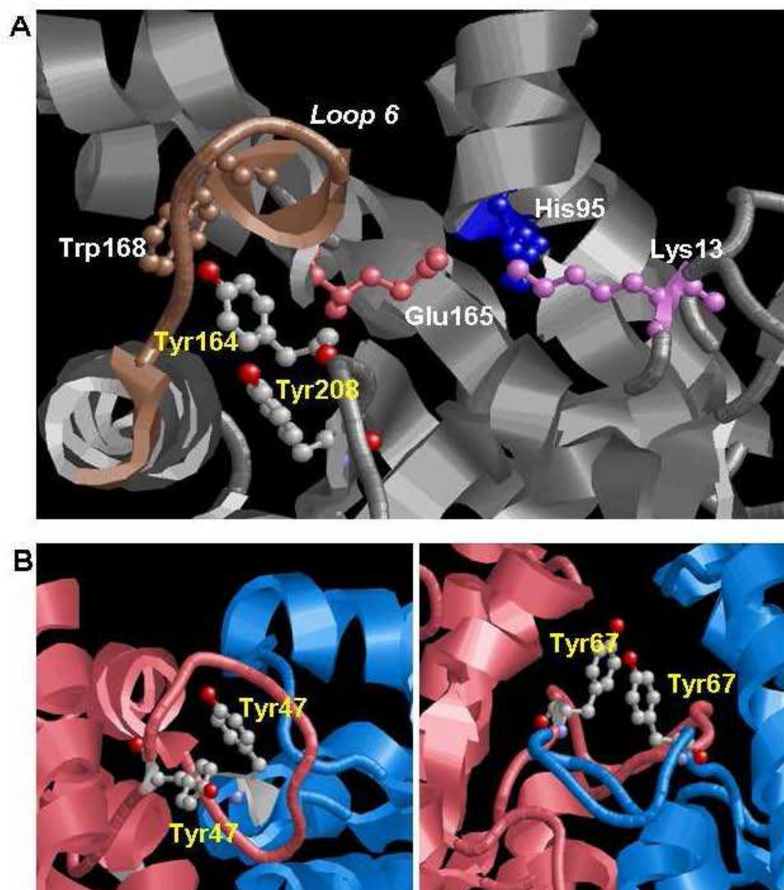

Figure 5

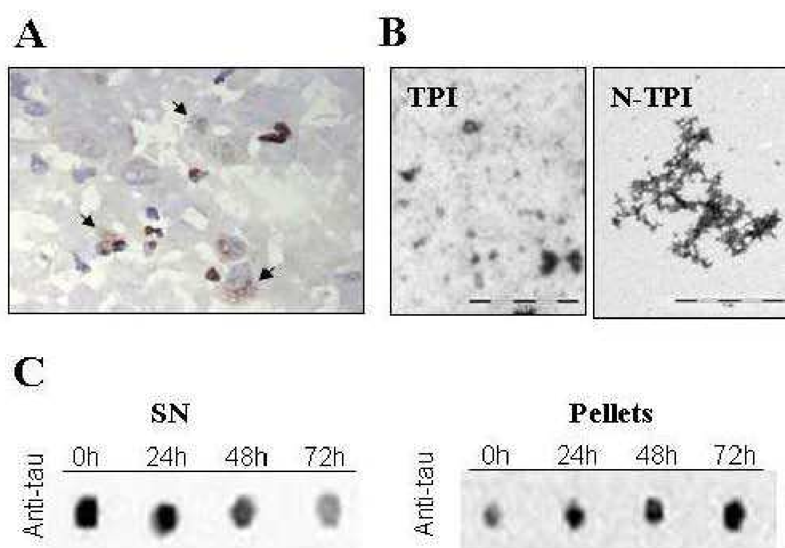

Figure 6