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 on fibrinogen in brain ischemia

Gerard ILL-Raga

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DIRECTOR DE LA TESI

Dr. Francisco José Muñoz López

Departament de Ciències Experimentals i de la Salut

UNIVERSITAT POMPEU FABRA

En el record del meu avi Ramon Raga Soriano,

Pel Daniel ILL i l'Enca Raga,

Para a Carmem e a Júlia,

vocês são a minha bênção nessa vida

Agraïments

Al Paco Muñoz,
per haver-me donat la mà i mostrat el camí.

Al Chema Fernández i al Miguel Valverde,
que han estat referents i suport.

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per l'agradable atmosfera de treball que hem sabut crear entre tots.

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La vostra companyia quotidiana ha estat un veritable bàlsam.

A la Marta ILL,
Que acabi aviat els seus estudis per poder salvar el país de tanta misèria política.

A les meves àvies,
Amb tot l'afecte.

Als meus amics; pels indissolubles llaços de l'amistat que ens uneix.

"Nosaltres els catalans creiem que hem de posar els peus molt bé a terra si volem saltar enlaire. Com que jo, una vegada i una altra, sempre torno a tocar de peus a terra, finalment he après a saltar molt més amunt"

Joan Miró

Abstract

Nitric oxide (NO) is a neurotransmitter involved in memory processes. Currently, the only recognized physiological signalling pathway controlled by NO is the activation of guanylyl cyclase. In this thesis, we propose an alternative NO-signalling pathway that involves the Heme-regulated eukaryotic initiation factor-2α kinase (HRI) and eIF2α phosphorylation. We have found that the enzyme BACE1, a key protein in Alzheimer's disease (AD), is controlled by this novel pathway. This pathway would be involved in the physiology of memory formation and learning processes. We have also studied how an external stress factor, the Herpes Simplex Virus 1, can disrupt this cascade leading to a pathological increase in BACE1 and amyloid ß-peptide (Aß) production. Aß aggregates forming fibrils that generate free radicals. These react with NO producing peroxynitrite, which contribute to AD progression. Since NO turns toxic when produced in a pro-oxidant environment we have also studied the effect of peroxynitrite in Stroke.

Resum

L'òxid nítric (NO) és un neurotransmissor involucrat en processos de memòria. Actualment, l'única cascada de senyalització fisiològica controlada per NO consisteix en l'activació de la guanilat ciclasa. En aquesta tesi, en proposem una d'alternativa que inclou la *fosforilació de* eIF2α per la *Heme-regulated eukaryotic initiation factor-2α kinase (HRI)*. Hem mostrat com l'enzim BACE1, una proteïna clau en la malaltia d'Alzheimer (AD), és controlat per aquesta nova cascada de senyalització, que podria estar involucrada en la fisiologia de l'aprenentatge i la memòria. També hem estudiat com un factor d'estrès extern, l' Herpes Simplex Virus 1, pot pertorbar aquesta cascada donant lloc a increments patològics en BACE1 i pèptid ß-amiloide (Aß). L'Aß agrega formant fibril·les que generen radicals lliures. Aquests reaccionen químicament amb NO produint peroxinitrit, que contribueix a la progressió de l'AD. Pel fet que l'NO esdevé tòxic quan és produït en un entorn pro-oxidant, hem estudiat també l'impacte que el peroxinitrit té en l'ictus.

Prologue

Nitric oxide (NO) is a gaseous molecule synthesized from the amino acid arginine by the NO synthase (NOS) family of enzymes. NO is a molecule with pleiotropic effects in different tissues, being involved in crucial biological responses such as vasodilatation and immune reactivity. Furthermore, NO is also an important neurotransmitter with especial relevance in learning and memory processes. After being synthesized in synaptic terminals, NO diffuses to pre-synaptic compartments and neighbouring cells where it starts signalling cascades. The classical pathway activated by NO in neurons promotes a raise in the secondary messenger cyclic GMP (cGMP) by the activation of the soluble guanylyl cyclase (sGC). NO allosterically activates sGC by binding to the heme group present in this enzyme. Based in the same principle of heme-mediated allosterical activation by NO, we have investigated an alternative signalling pathway in which NO activates the Heme-regulated eukaryotic initiation factor(eIF)2α (HRI) kinase promoting a raise in the levels of phosphorylated eIF2α. The phosphorylation of this factor is part of eukaryotic cell's general response to stress, but is also involved in the activation of specific gene synthesis at the level of translation. Such is the case of genes harbouring special features in its 5'untranslated regions (5'UTR). In this regard, we have studied BACE1 (ß-site APP cleaving enzyme type 1) regulation by NO, in a pathway involving HRI activation, eIF2α phosphorylation and BACE1 translation through the release of its 5'UTR translational brake. BACE1 is the protease responsible of the ß-secretase activity which generates the amyloid ß-peptide (Aß) through the proteolysis of the Amyloid Precursor Protein (APP). Aß production is the upstream event driving Alzheimer's disease (AD), the most devastating dementia in the current times characterized by memory loss and cognitive

deterioration. BACE1 and the other components of the amyloidogenic cascade were discovered for its implication in the pathology after the Aß-peptide was identified as the major component of senile plaques. Based on the results presented in this thesis, we think that the amyloidogenic cascade is not just a focus of neuronal stress but it is intimately linked to memory formation processes. The fact that a neurosignal such as NO controls BACE1 translation might contribute to uncover the physiological significance of APP (amyloid precursor protein) cleavage in the context of learning and memory. Furthermore, numerous cellular stresses commonly linked with AD (oxidative stress and calcium dyshomeostasis among others) can induce eIF2α phosphorylation through the activation of specific stress-responsive eIF2α kinases. This implies that the delicate physiological equilibrium of eIF2α phosphorylation controlled by NO signalling can be interrupted by external stress factors. In this regard, we have investigated how the Herpes Simplex Virus type 1 (HSV1), well-known to be associated with AD, can promote amyloidogenesis through the activation of the double-stranded RNA activated protein kinase (PKR), a kinase that phosphorylates eIF2α causing BACE1 upregulation. Finally, we have adressed the study of the pathological events that can be triggered by NO when it is produced in excessive amounts in a pro-oxidative environment, something that leads to the formation of the noxious peroxynitrite anion ($ONOO^-$) as a result of chemical combination of NO and supeoxide anion ($O_2^{\cdot-}$). Peroxynitrite, among other harmful effects, nitrates tyrosine residues of proteins —a process termed *nitrotyrosination*— impairing the function of the targeted protein. We have studied the role of peroxynitrite in Aß induced cytotoxicity, identifying different mechanisms to avoid the cell damage. Moreover, we have investigated the nitrotyrosination of fibrinogen after an ischemic stroke, a condition in which NO is released in a pro-oxidant environment.

As fibrinogen is a plasmatic protein essential in hemeostasis, structural changes originated by its nitrotyrosination might be relevant in stroke progression.

Index

Abbreviations

ACh	acetylcholine
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase
AICD	APP intracelular domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APOE4	apolipoprotein E4
APP	amyloid precursor protein
ASP-2	aspartyl protease 2
Aß	amyloid ß-peptide
BACE1	ß-site APP cleaving enzyme type 1
CAD	coronary artery disease
cGMP	cyclic GMP
CNS	central nervous system
CSF	cerebrospinal fluid
CTFß	C-terminal ß-fragment
CTFα	C-terminal α-fragment
EDRF	endothelial-derived relaxing factor
eIF2α	eukaryotic initiation factor 2-α
eNOS	endothelial nitric oxide synthase
FAD	Familiar AD
FGA	fibrinogen Aα chain gene
FGB	fibrinogen Bß chain gene
FGG	fibrinogen γ chain gene
FSF	fibrin stabilizating factor
GCN2	general control non-derepressible 2 kinase
GTP	guanosine-5'-triphosphate

HC-VSMC	human brain vascular smooth muscle cells
HRI	Heme-regulated eukaryotic initiation factor(eIF)2α kinase
HSV1	Herpes Simplex Virus type 1
HUVEC	human umbilical vein endothelial cells
iNOS	inducible or immunological nitric oxide synthase
LTD	long term depression
LTP	long term potentiation
mtNOS	mitochondrial nitric oxide synthase
NFTs	neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NOS	NO synthase
OGD	oxygen-glucose deprivation
ORF	open reading frame
PERK	doublestranded RNA-activated protein kinase-like ER kinase
PI3K/Akt	phosphatidylinositol 3-kinase
PKR	double-stranded RNA-activated protein kinase
PS1	presenilin 1
PS2	presenilin 2
PSD-95	postsynaptic density protein 95
ROS	reactive oxygen species
sAPPß	soluble APPß ectodomain
sAPPα	soluble APPα ectodomain
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside

SOD	superoxide dismutase
uAUGs	upstream initiating codons
5'UTR	5'untranslated region

Molecular formulas

$\cdot NO_2$	Nitrogen dioxide
$ONOO^-$	Peroxynitrite anion
$O_2^{\cdot -}$	Supeoxide anion

1. Introduction

1.1. The NO

1.1.1. NO discovery

"(...) la qüestió no està aclarida del tot perquè en els vasos, fins a la data no s'hi ha pogut trobar cap múscul dilatador que pugui posar-se en moviment a través dels nervis vasomotors".

Conseller àulic Behrens, responent a la curiositat de Hans Castorp sobre qüestions de Fisiologia al sanatori de Davos. *La muntanya màgica,* Thomas Mann.

For over 160 years, the vasodepressor action of organic nitrates and nitrites has been known and sodium nitroprusside (SNP), an organic nitroso compound that spontaneosly releases nitric oxide (NO) in aqueous solution, has been employed sporadically as a hypotensive drug, despite of the physiological mechanisms underlying this action were completely unknown.

The role of NO in blood vessel and macrophage physiology was first appreciated in the 1980s. Furchgott and Zawadzki discovered that the vasodilating effect of acetylcholine (ACh) in isolated preparations of blood vessels *in vitro* depended on the integrity of the intimal surface of the blood vessel preparation[1]. They demonstrated the requirement of endothelial cells for the relaxation phenomena and postulated the existence of a substance elaborated and released by the endothelium that would enter the smooth muscle cell to relax it. This endothelial-derived relaxing factor (EDRF) was later shown to be NO [2,3].

The first evidence of neuronal NO comes from the work of Garthwaite and colleagues [4]. They reported the presence of a substance with EDRF activity in the cerebellum after N-methyl-D-aspartate receptor (NMDAR) activation, suggesting a role for NO as the intracellular signalling

molecule that would link NMDAR activation and cGMP production in the post-synaptic compartment.

1.1.2 Chemical structure

NO is a small gaseous molecule of 115 pm of diameter formed by a double covalent bond between one oxygen and one nitrogen atoms (Fig.1).

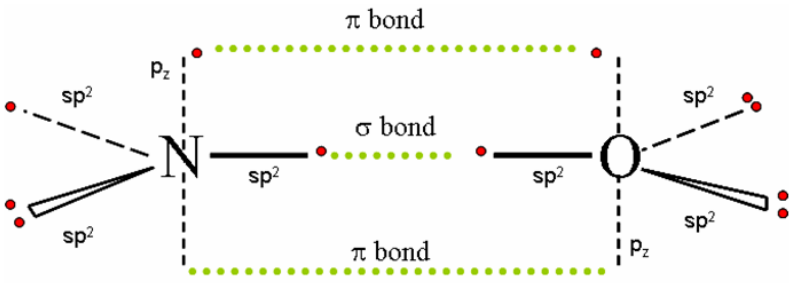

Figure 1. *Molecular structure of NO*. Red dots represent valence electrons; Green dotted line represent molecular chemical bondings; sp^2: Molecular orbitals resulting from the linear combination of atomic orbitals 2s, $2p_x$ and $2p_y$.

One of the sp^2 orbitals belonging to the nitrogen atom lacks one electron, endowing the molecule with a high chemical reactivity commonly attributed to free radicals. NO is an electrically neutral molecule which can diffuse through lipidic bilayers highly impermeable to ions. In fact, NO is one of the most diffusible molecules ever reported, with a tissue diffusion estimated at 848 square micrometers per second[5]. It is a key property of NO regarding its role as secondary messenger. On the other hand, its relatively short half life (3-5 s) due to its high reactivity counterpart this pronounced tendency to spread [6].

1.1.3. The NOS

NO is mostly produced by the NOS enzyme family. There are four major members of the NOS family: neuronal (nNOS), endothelial (eNOS), inducible or immunological (iNOS) and mitochondrial (mtNOS). The former is an isoform of nNOS localized in the inner mitochondrial membrane[7]. This family of enzymes catalyzes the chemical reaction responsible for NO generation by the oxidation of L-Arginine in the presence of NADPH to deliver NO and citrulline (Fig. 2).

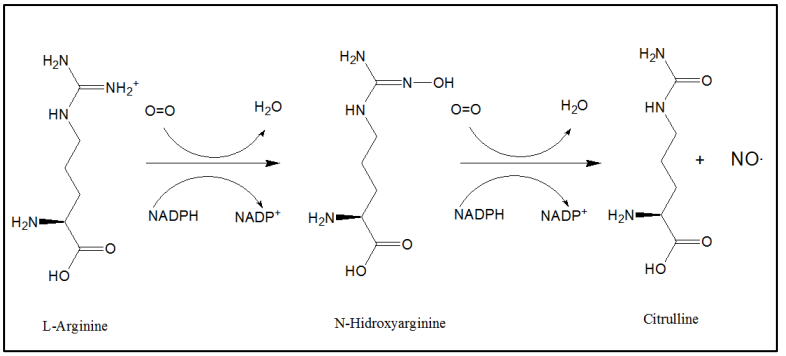

Figure 2. *NO synthesis by NOS*

The NOS biology is complex at many levels: different genes in different chromosomes per each NOS, high promoter complexity, wide splicing diversity generating diverse isoforms, and numerous allosteric activators. Regarding this last consideration, eNOS (NOS III) and nNOS (NOS I) contain a Ca^{+2}/calmodulin binding domain which makes them responsive to calcium [8], whereas iNOS (NOS II) is Ca^{+2}/calmodulin independent and its regulation depend on *de novo* synthesis [9].

The iNOS is codified by a gene located at chromosome 17 [10]. iNOS is expressed in immune and glial cells, although the presence of iNOS has

been also reported in other tissues [11-13]. Its activity depends on *de novo* synthesis from DNA, being NF-κβ and IRF-1 (interferon regulatory factor 1) the main transcriptional activators [14,15]. Its function is associated with the immunological response and the increase in the local vasodilatation.

The nNOS is codified by a gene at chromosome 12 [16]. It is found mainly in developing and mature neurons, although it has also been found in other tissues [17-27]. nNOS gene has the most complex human promoter ever described. Moreover, mRNA splicing generates four diferent isoforms[28]: nNOSα and nNOSμ (anchored to subcellular structures by a PDZ domain), and nNOSγ and nNOSβ (thought to be cytoplasmatic). PDZ domains bind to the postsynaptic density protein 95 (PSD-95) [29] and to the related protein PSD-93 [30]. It explains the tight interaction between nNOS and NMDAR, as NMDAR are also known to be associated with PSD-95 [31]. The function of nNOS in glutamatergic transmission has been associated to the neuromodulator role of NO especially in memory and learning processes by its contribution to the trigger and maintenance of long term potentiation [32,33].

The eNOS is codified by a gene located at cromosome 7 [34]. eNOS is mainly expressed in the endothelium although it has been detected in some other cell types [35-38]. eNOS undergoes important post-translational modifications, such as myristolation of glycine-2 and palmytoylation of cystein-15/26. These modifications allow the binding of eNOS to caveloae, where it remains inactivated by its interaction with caveolin-1 [39]. Activation of eNOS depends on phosphorylation by phosphatidylinositol 3-kinase (PI3K/Akt) and the binding of Ca^{+2}/calmodulin [40,41] Activation of eNOS is crucial for blood pressure control due to the miorelaxing properties exerted by NO on smooth muscle cells from the vascular wall.

1.1.4. Physiological roles: cellular effects of NO

NO binds easily to the heme group of proteins, such as guanylyl cyclase. In fact, the most important NO signalling pathway is the activation of GC, but there exist other GC independent effects mediated by NO.

cGMP dependent effects: The heme group present in GC contains a central iron that can bind NO. NO binding triggers a conformational change that activates the catalysis of guanosine-5'-triphosphate (GTP) in cGMP [42]. cGMP is a secondary messenger that activates PKG I, PKG II, and modulates the activity of certain phosphodiesterases of cyclic nucleotides [43-46].

cGMP independent effects: NO can bind to biomolecules by different mechanisms. Nitrosation is a process consisting in the addition of a NO group to organic molecules without producing any change in the substrate charge. *C*-nitroso, *N*-nitroso, *O*-nitroso or *S*-nitroso derivatives are produced by this process [47]. S-nitrosation is a post-translational regulatory mechanism that usually decreases the activity of the targeted protein. The main targets for S-nitrosation are kinases and enzimes although it has been recently demostrated that NO can nitrosylate membrane proteins such as the NMDAR or the Na^+/K^+ ATPase [48,49]. Interestingly, S-nitrosation on eNOS prevents its dimerization and subsequent activation, in a sort of autorregulatory mechanism in order to control NO production [50].

1.1.5. Pathological roles: peroxynitrite and nitrotyrosination

NO is also able to induce pathological processes. It is mainly through the formation of the highly reactive peroxynitrite anion ($ONOO^-$). When NO

is produced in a pro-oxidant environment, it reacts with superoxide anion to deliver peroxynitrite [51]. This reaction cannot be avoided by superoxide dismutase (SOD) since the affinity of $O_2^{\cdot-}$ is ten times higher for NO than for SOD [52,53]. Peroxynitrite induces the protein nitrotyrosination. It consists on the addition of a nitro group (NO_2) into tyrosine residues to deliver 3-nitrotyrosine (Fig. 3). It is not a stochastic process, as it has been determined that under inflammatory conditions only one to five of every ten thousand tyrosines are nitrotyrosinated [54]. It has been proposed that the proximity of negatively charged residues increase the susceptibility of tyrosines to be nitrated [55].

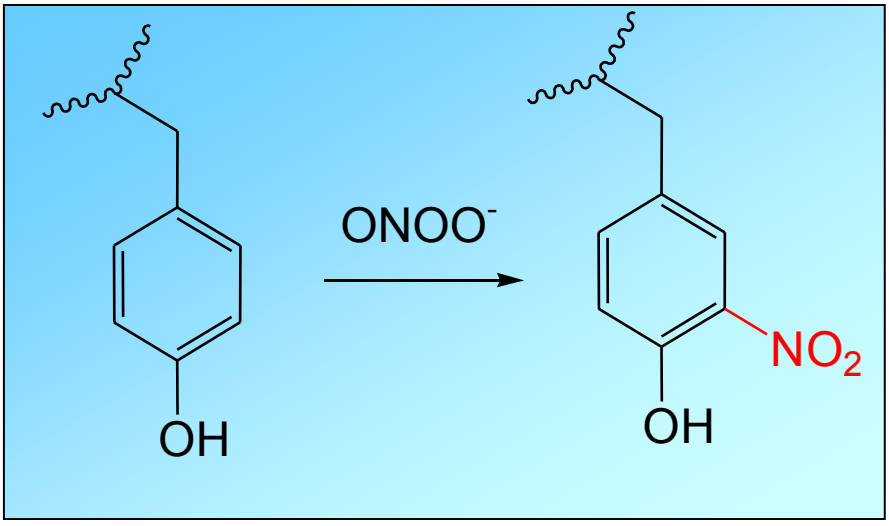

Figure 3. *Nitrotyrosination process*

The addition of the nitro group can produce a change in the conformation of the protein or generate undesired spatial constrains [56,57]. Nitrotyrosination is a post-translational modification that normally alters

pathologically the targeted protein leading to a loss of the physiological activity [58]. It can also inhibit phosphorylation sites in tyrosine dependent signalling pathways. Some authors have suggested that nitrotyrosination can be a labeling mechanism that directs old proteins to degradation. Nevertheless, another scenario of potentially greater biological significance is the modification of the protein function with controversial opinions about an inhibitory effect or a gain-of-function [59-63]. Such scenario will be considered in the present work regarding the nitrotyrosination of fibrinogen, which makes the molecule more prone to clot formation [64].

Many neurodegenerative diseases such as brain ischemia, AD, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and Huntington's disease are linked to an increase in reactive oxygen species (ROS) production [65]. In all of these pathologies there have been detected high levels of nitrotyrosination [66-69].

1.1.6. NO and neuronal plasticity

NO is a gaseous neurotransmitter/neuromodulator that is not pre-synthesized and stored in synaptic vesicules at the pre-synaptic compartment as the classical neurotransmitters. NO is synthesized immediately and rapidly liberated within few seconds, diffusing to the nearby post-synaptic neurons. NO was discovered in brain as the intracellular transmitter that linked NMDAR activation to the production of cGMP [4]. Indeed, the NO receptor is guanylyl cyclase, an intracellular heme-enzyme that requires the binding of NO to its heme group for the allosterical activation of its enzymatic activity.

Depending on the circuit, NO can be produced pre-synaptically or post-synaptically. In peripheral nitrergic nerves NO derives from pre-synaptic axon terminals, in which Ca^{+2} entry through Ca^{+2} voltage dependent

channels activates NO synthesis and its immediate release. However, in most brain regions, NO is produced post-sinaptically as the nNOSα isoform interacts intracellularly with the NMDAR through a PDZ domain (Fig. 4). The interest of NO in long term changes of synaptic strength comes from its diffusible nature and its link to the NMDAR, which in turn is related to synaptic plasticity through NMDAR-mediated calcium influx. All this make NO an appealing retrograde messenger able to coordinate alterations in neurotransmitter release, informing about the actions of NMDAR to the pre-synaptic terminal in a process thought to be relevant for long term potentiation and memory consolidation [70,71]

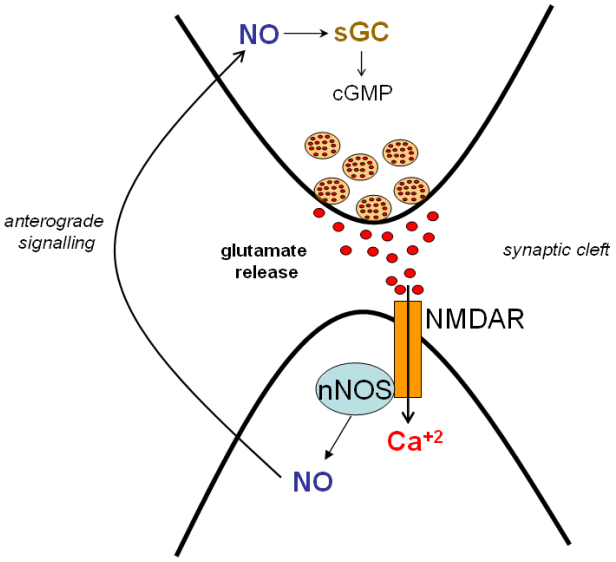

Figure 4. *NO anterograde signalling*

The advantage of NO as messenger is that generates the same signalling in both synaptic compartments, contributing to an harmonization of the signalling in both compartments of the synapse, something thought to be

required for proper synaptic transmission. Interestingly, NO plays a role in modulating synaptic strength at many synapses through the nervous system[72]. Depending on the intracellular cascades triggered by NO at a particular synapse, NO can induce opposite effects: long term potentiation (LTP) or long term depression (LTD), evidentiating its important role in memory formation[73,74].

Finally, NO mediates receptor-dependent growth of pre-synaptic protusions and the remodeling of pre-synaptic varicosities in hippocampal slice cultures[71], a process crucial in synaptogenesis and spine formation. In the light of the results presented in this thesis, we speculate with the possibility that NO participates in synaptic remodelling through the modulation of the amyloidogenic cascade, which is mainly conceived, today, only under the frame of pathology.

1.2. The physiopathological role of APP

1.2.1. Introduction to AD

Historically, senile dementia was not considered as a disease but conceived as a normal process of the aging mind. The work by Alois Alzheimer at the beginning of 20th century contributed decisively to change the spread settled notion that mental decline occurred inevitably in old age.

Dr. Alois Alzheimer had a patient, Auguste D., who debuted with a progressive “pre-senile dementia” at the relatively young age of 51. Her presenile dementia rapidly turned fulminant. Auguste D. developed a rapid loss of memory and became disoriented in time and space. She died four and a half years after the onset of the disease. Alois Alzheimer examined her brain post-mortem and reported the findings of his studies at

the 37th meeting of South-west German Psychiatrists that took place in Tübingen, Germany, in 1906. In the next year, Alois Alzheimer published a paper entitled "Über eine eigenartige Erkrankung der Hirnrinde*" (About a peculiar disease of the cerebral cortex)*[75] in the journal Allegemeine Zeitschrift für Psychiatrie und Psychisch-Gerichtliche Medizin. In this paper Alois Alzheimer describes an atrophic brain with unusual deposits in the cortex and prominent neurofibrillary pathology. Based on Alzheimer's descriptions, it seems that Auguste D did not have a classical AD, but a dementia with Lewy bodies or a frontotemporal dementia. Nevertheless, still nowadays we use these two hystopathological changes that were originally described in that patient to make the proper diagnosis of AD. The "unusual cortex deposits" are known today as *senile plaques*, constituted by the extracellular Aß deposits, and the "prominent neurofibrillary pathology" is referred under the name of *neurofibrillary tangles* (NFTs), intracellular deposits of the microtubule associated protein *Tau* [76,77]. Therefore, Alzheimer's publication in 1907 was a landmark on the understanding of the disease that today bears his name, as he ascertained to describe both senile plaques and NFTs, which are considered currently as the main hystopathological AD hallmarks. It was in 1910 when, at the suggestion of Kraeplin, who had been Alzheimer's mentor in Munich, *pre-senile dementia* was renamed AD [78]. One year later, Alzheimer published a review in which he exposed that there were little or no difference between pre-senile dementia (or *AD*) and senile dementia [79]. This was a crucial advance that opened the way towards the understanding of senile dementia as a disease that could be potentially treated clinically.

1.2.2. General characteristics of AD

AD is the most common form of human dementia affecting more than 18 million people worldwide. Initially, sufferers experience severe memory deficits and cognitive decline as the consequence of the neuronal damage at the hippocampal formation. As the disease advances, neuronal damage generalizes at the prefrontal cortex and as a result, speech and comprehension are severely impaired, and the symptoms progressively worsen over 5 to 10 years. The brains of AD sufferers are characterized by two pathological features: senile plaques and neurofibrillary tangles. The former consistof amyloid ß-protein[76,77], a peptide generated by the enzymatic cleavage of an integral type I transmembrane glycoprotein called amyloid precursor protein, and the latter consists mainly of abnormally phosphorylated aggregates of the microtubule-associated protein tau.

AD can be classified into two different types according to the onset time of the symptoms: Familiar AD (FAD) and sporadic AD. FAD constitutes an early onset form of the disease. It appears before the age of 65 years old and it accounts for less than 3% of all AD cases [80]. FAD is caused by unknown causes but about 5% of these patients have mutations in APP or in presenilins (PS1, PS2) genes. The fact that these mutations are related to Aß cleavage reinforces the amyloid hypothesis for AD etiology. Nevertheless, sporadic AD is the most common form of AD. In sporadic AD, the onset of the disease occurs after 65 years old without any mutation in genes related to the amyloidogenic cascade. The underlying cause of sporadic AD is still unknown, altough there are some polymorphisms which are known to increase the probability to develop AD, for instance in the apolipoprotein E4 (APOE4) and PKR genes [81,82]. In fact, the principal risk factor for developing sporadic AD is age, which doubles the incidence of the disease every 5 years after 65 years of age [83]. Nevertheless, data on centenarians shows that AD is not necessarily the outcome of aging [84].

1.2.3. The amyloid hypothesis

After the Aß was found to be the main component of the senile plaques [77], the Aß coding-gene ─latter on named Amyloid Precursor Protein─ was cloned and localized in chromosome 21 [85-88]. The observation that trisomy 21 (Down's syndrome) invariably causes AD dementia [89] led to the formulation of the amyloid hypothesis of AD. According to this hypothesis, Aß accumulation in the brain is the upstream event that triggers AD pathogenesis. Albeit the inherited forms of AD are the minority (~3%), the subsequent identification of AD-causative mutations in the APP gene [90-92] and in the presenilins (PS1-2) genes [93-95] provided a strong genetic framework for the emerging amyloid hypothesis. Presenilins are the catalytic subunit of the gamma-secretase complex[96,97]. PS1 and PS2 mutations change Aß metabolism shifting it to the formation of the more aggregant $Aß_{1-42}$. There are also mutations in the APP sequence that turn Aß more prone to aggregate (*Dutch* and *Arctic* mutations) [98-100]. Other mutations make APP more accessible to the ß-secretase increasing the Aß production (Swedish mutation)[101]. These genetic evidences also pointed towards ß-amyloid production and agregation as crucial events in AD pathogenesis.

1.2.4. APP processing and Aß production

The Aß length varies from 38 to 46 amino acids[102]. $Aß_{1-40}$ is the predominant secreted Aß isoform. The longer isoforms are minoritary. Nevertheless, these longer isoforms, especially $Aß_{1-42}$, are more hydrophobic and as a consequence they are more amyloidogenic –as they have enhanced aggregative properties. In addition, the longer Aß isoforms

are considered to have an important role in the seeding of Aß fibrils and in the nucleation of amyloid plaques [103,104].

Altough the highest expression of APP is found in brain and kidney, APP is a ubiquously expressed glycoprotein whose function has been related with cell adhesion to extracellular matrix and cell to cell interactions [105,106].

There are a number of APP isoforms that differ in length. In the brain, the most common isoforms are APP_{695} and APP_{714} [107]. The extracellular region of APP is subdivided in two different domains, E1 and E2. The E2 domain contains a dimerization motif that may bind proteoglycans to the extracellular matrix [108]. The E1 domain contains several subdomains including a metal binding motif, a growth factor-like domain and a serine protease inhibitor. This last subdomain is absent in the APP isoform present in brain [109]. APP undergoes extensive post-translational modifications including glycosilation, phosphorylation and tyrosine sulfation [110].

APP can be processed via two alternative pathways, the amyloidogenic pathway and the non amyloidogenic pathway (Fig. 6).

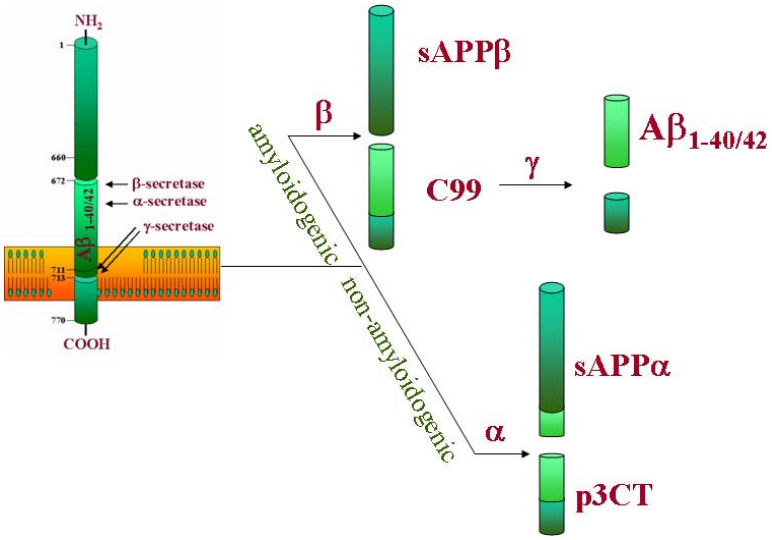

Figure 6. *APP proteolytic processing*

In the non-amyloidogenic pathway APP is cleaved within the Aß sequence by an α-secretase. As a consequence, a soluble ectodomain named sAPPα is released and a C-terminal fragment (CTFα) remains anchored to the membrane. When APP undergoes the non-amyloidogenic pathway, the Aß formation is avoided. In the amyloidogenic pathway, APP undergoes a proteolytic cleavage in its N-terminal domain by ß-secretase. Similarly with the non-amyloidogenic pathway, a soluble sAPPß ectodomain is released and a C-terminal fragment (CTFß) remains anchored to the membrane. Both CTFα and CTFß are cleaved within the transmembrane domain by a γ-secretase to produce the short peptide p3 from CTFα and Aß from CTFß, in addition to the release of the APP intracelular domain (AICD) from both CTFα and CTFß [96]. p3 is not involved in amyloidogenesis [111]. The AICD produced in both pathways has been proposed as a transcription factor[96]. AICD is known to bind some intracellular target proteins when released from APP and it might be

involved in different cellular events such as apoptosis, neuronal growth and regulation of gene expression [112].

1.2.5. The secretases

Before the ultimate proteases cleaving the APP at different sites were isolated and identified, the term "secretase" referred to the various enzymatic activities acting on the three APP cleavage sites: alpha, beta and gamma. Currently, some of the enzymes responsible for these secretase activities have already been identified.

1.2.5.1. The α–secretase activity

The α-secretase activity is responsible for the constitutive secretion of sAPPα in the brain. In most types of peripheral cells the non-amyloidogenic pathway driven by α-secretase is predominant [113] and even in neuronal cells, ß-secretase activity only cleaves ~10% of total APP [111], implying that the majority of APP molecules in neurons are recruited to the non-amyloidogenic pathway. However, the proportion of APP cleaved by the amyloidogenic pathway increases with age [114]. α–cleavage mainly occurs after Lys-16 [115], interrupting the Aß sequence at one third of its extension and rendering subproducts without any fibrillogenic property [111,116]. Multiple studies suggest that the α-cleavage is mediated by members of the ADAM (a disintegrin and metalloproteinase) family of proteases [117]. These enzymes are widely expressed, contain adhesive and metalloprotase domains, and play an important role in the release of multiple cell surface proteins such as growth factors, cytokines and receptors [118]. Altough ADAM 9, 13 and 17 have been proposed as plausible alpha-secretases, ADAM10 is likely to be the best candidate for

alpha-secretase activity [119-122]among the members of the ADAM family of proteins.

1.2.5.2. The γ-secretase activity

The γ-secretase cleaves APP at the C-terminus end of the Aß sequence. The membrane anchored C-terminal fragments produced by the previous cleavage by α- or ß-secretases (CTFα and CTFß) are the substrates of γ-secretase activity. This activity renders different Aß species, which differ in length from 38 to 46 amino acids, but predominantly $Aß_{1-40}$ and $Aß_{1-42}$. Interestingly, γ-secretase cleavage determines the ratio of $Aß_{1-42}/Aß_{1-40}$ [94], a key factor in Aß aggregation in AD [123].

The γ-secretase cleavage occurs within the lipid membrane bilayer through a regulated intramembrane proteolysis [102]. This proteolysis within the hydrophobic environment of a membrane has attracted general biological interest, as all other proteases need the activation of water molecules to catalyze the cleavage of the peptidic bond existing among the individual amino acid residues of proteins.

Active γ-secretase is a high molecular weight complex composed by at least least four proteins: presenilin, nicastrin, Aph1 and Pen-2. Presenilin is the catalytic subunit responsible for the intramembranal aspartyl protease activity [95]. Mutations in the presenilin genes are associated with FAD. γ-secretase is also involved in the processing of a subset of type I transmembrane proteins such as the Nectin-1 receptor [124], LRP [125], E-cadherin [126] or Notch [96]. Therefore, a total gamma-secretase inhibition as a therapeutical approach in AD is not considered for its important implication in surface receptor-linked signalling pathways.

1.2.5.3. The ß-secretase activity

1.2.5.3.1. BACE1 description

The ß-secretase activity renders sAPPß and the membrane-anchored CTFß that will be later processed by γ-secretase to deliver Aß. BACE1, an aspartyl protease belonging to the type I integral membrane protein family is the beta-secretase [127]. Alternative names for BACE1 are ASP-2 (aspartyl protease 2) or memapsin 2 (membrane-anchored aspartyl protease of the pepsin family). BACE1 is constituted by 501 amino acids, containing two aspartyl active sites motifs in the N-terminal catalytic domain, a 17 residue trans-membrane domain and a short C-terminal tail. BACE1 has an homologue protein, BACE2, which has a different specificity in the cleavage of APP and is expressed at much lower levels in the brain than BACE1 [128]. Indeed, BACE1 expression is tightly regulated, both at the transcriptional and at the translational level. Multiple putative transcription factors can bind the promoter region of BACE1 gene [129,130]. In addition, BACE1 mRNA translation is activated by eIF2α phosphorylation at Serine 51 [131]. This translational activation-effect in response to p-eIF2α is caused by the particularities of BACE1 5'UTR region [132-135].

1.2.5.3.2. BACE1 in AD

BACE1 is a good target for pharmacological inhibition [136]. Nonetheless BACE1 has other substrates such as Neuregulin 1, a surface receptor needed for normal neuronal-glial signalling and peripheral neuron myelinization [137,138]. Furthermore, BACE1 activity upon APP could be essential for synaptic plasticity, learning and memory [139-141]. According to these facts, BACE1 activity in the brain must be regarded as physiological, being dysregulated in the context of AD and

amyloidogenesis. In this sense, the effort should be directed to the understanding of BACE1 dysregulation in AD rather than to the complete inhibition of BACE1 activity, which could imply detrimental effects. Nevertheless, in sporadic AD the total BACE1 content and activity is markedly upregulated and strategies to reduce Aß production are necessary.

1.2.5.3.3. BACE1 translational control

The transcription of BACE1 gene is stimulated under conditions of oxidative stress [142,143] or hypoxia [144,145]. However, the levels of BACE1 mRNA found either in brains from AD patients [146,147] or in neurons surrounded by senile plaques in APP transgenic mice [148] are not elevated, discarding the possibility that a transcriptional effect on BACE1 gene can be responsible for the increased Aß production found in AD brains.

Indeed, the limiting step in the control of BACE1 expression is translation, not transcription. BACE1 mRNA bears a 5'UTR that avoids its translation under basal conditions. BACE1 5'UTR is 570 nucleotides long, contains four upstream initiating codons (uAUGs) and possesses a rich GC content which confers a secondary structure to that region of the mRNA. All these features prevent the ribosome from reaching the main AUG to start BACE1 polipeptide synthesis (Fig. 7).

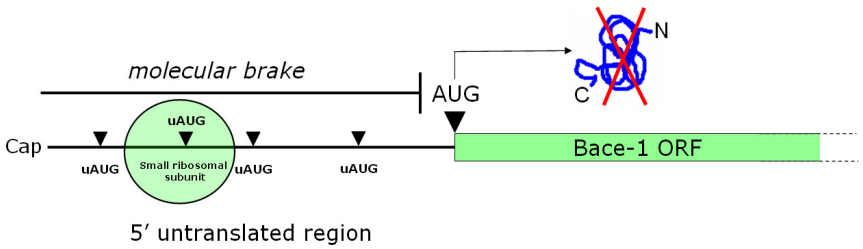

Figure 7. *BACE1 transcript leader.* BACE1 tanscript leader, alternatively named 5'untranslated region (5'UTR), prevents translation initiation of BACE1 protein under basal circumstances.

Under basal conditions, the ribosome gets stuck in one of the uAUG – especially the second one- and never gets to reach BACE1 open reading frame (ORF).

Therefore, the population of BACE1 mRNAs is translationally inactive [132-135] and the transcriptional up-regulation mediated through the action of transcription factors such as HIF-1 only contributes to the enlargement of a dormant cellular BACE1 mRNA pool.

The translational brake imposed by BACE1 5'UTR can be overcome by the phosphorylation of the eukaryotic initiation factor 2-α[131]. eIF2 is one among the large list of existing eukaryotic translation factors. eIF2 is a trimeric G protein that binds a transference mRNA coupled to Metionine (Met-tRNA) and a GTP molecule rendering a molecular complex known as *ternary complex* (Fig. 8).

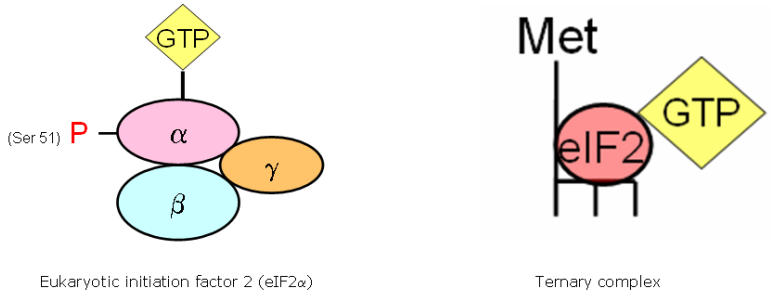

Figure 8. *eIF2 and the ternary complex*

The mission of this ternary complex is to load the initiating Met-tRNA to the ribosome to initiate mRNA translation, in a process that consumes the energy contained in the GTP, which consumes one rich energy phosphate rendering GDP. After one round of initiation is completed, eIF2 interacts with the guanine exchange factor eIF2B, which replaces the consumed GDP molecule for a new GTP molecule (Fig. 9).

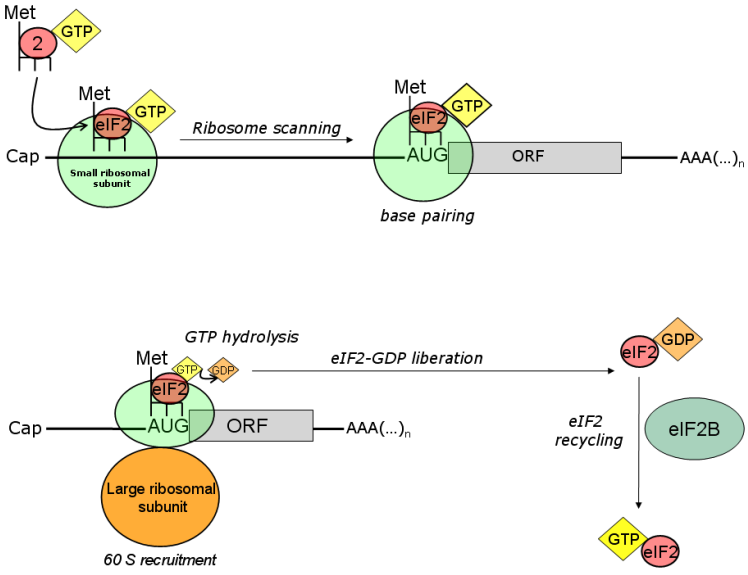

Figure 10. *eIF2 recycling*

When the alpha subunit of eIF2 is phosphorylated at Serine 51, the affinity for eIF2B becomes increased resulting in a blockade of eIF2B activity. As eIF2 is found in higher proportion in the cell when compared with eIF2B, the phosphorylation of a relative small fraction of eIF2α will result in the complete inhibition of eIF2B activity. As a consequence of the inhibition of eIF2B activity, the GTP-bound active ternary complex cannot be recycled and translation initiation is stopped.

The biological significance of eIF2α phosphorylation lies in the arrest of protein synthesis under conditions of stress in which the cell cannot maintain anabolic pathways. Examples of such stressful conditions are oxidative stress, nutrient deprivation, missfolded proteins, ER stress, viral infections etc. All these stresses are sensed by one of the four existing eIF2α kinases (HRI, GCN2, PKR and PERK) which phosphorylate eIF2α at Serine 51 blocking translation initiation [149] (Fig. 10).

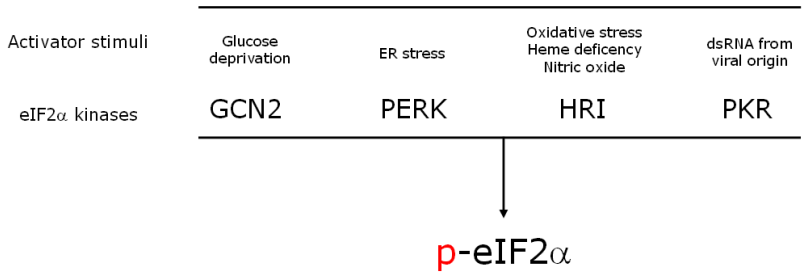

Figure 10. *The eIF2a kinases and its activator stimuli*

However, there is a special subset of genes that need to be translated to overcome the stressful situation. Such is the case, for instance, of GCN4, a

gene needed for the biosynthesis of aminoacids [150,151]. These gene types evolved to have a 5'UTR with special features that allow the translation of their mRNAs when the global mRNA translation is arrested as a consequence of eIF2α phosphorylation. Interestingly, BACE1 bears a 5'UTR with the hallmarks of this responsiveness to p-eIF2α: longer than 200 bp, with rich GC content and containing uAUGs.

The paradox effect observed in these mRNAs containing a 5'UTR of such characteristics can be understood in figure 11.

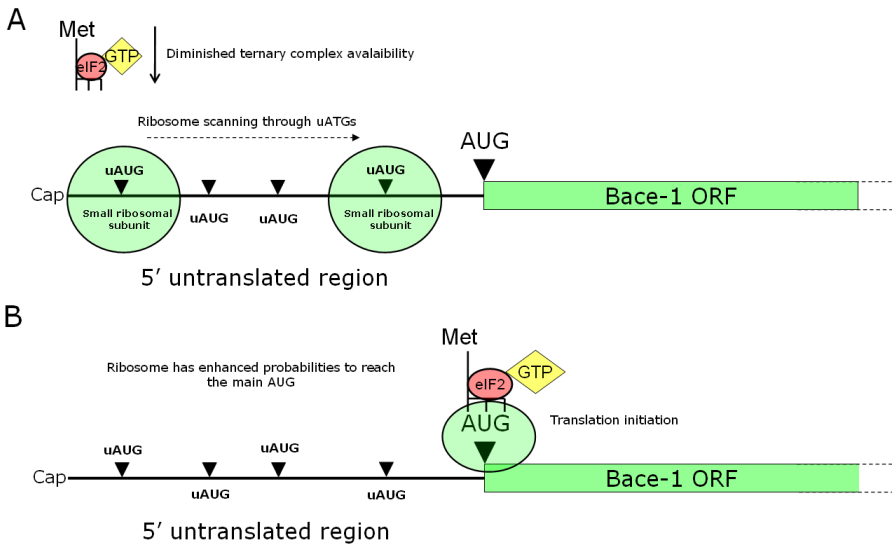

Figure 11. *Translation initiation at BACE1 mRNA.* BACE1 translation is favored by a decrease in the availability of the ternary complex.

When eIF2α is phosphorylated, there is less ternary complex availability. As a consequence, the opportunities for the ribosome to get stuck in one of the *fake* uATGs are reduced. Therefore, the chances to reach to the main ATG and start protein synthesis become effective.

The study of the characteristics of BACE1 5'UTR revealed its role in inhibiting BACE1 mRNA translation [132-135] and clearly pointed towards a translational activation mediated by eIF2α phosphorylation. Last year, it was definitely demonstrated that BACE1 translation is activated in response to eIF2α phosphorylation [131].

It is generally believed that this 5'UTR translational regulation in the case of BACE1 reveals a stress-response physiological role for the protease, as it is the case of its 5'UTR homologues. Nevertheless, we speculate that this translational control accomplishes a specialized role in the context of neurotransmitter signalling in neuronal terminals.

1.2.6. AD and nitro-oxidative stress

The involvement of NO in AD pathology is based on its pathologycal properties when NO is produced in an oxidative stress environment, as explained formerly. There are solid evidences relating AD pathophysiology with oxidative stress [152,153]. Soluble Aß turns neurotoxic when it misfolds in ß-sheet fibrils [154] and Aß fibrils produce hydrogen peroxide [155-157], a diffusable molecule than can cross biological membranes and start an intracellular cascade of reactive oxygen species production, spreading the toxic effect generated by Aß fibrils away from the original focus of Aß deposition. In such escenario, the superoxide anion produced intracellularly through hydrogen peroxide action reacts with NO to deliver peroxynitrite anion, a highly reactive molecule that nitrates the tyrosine residues of proteins impairing its biological functions [158].

The activation of NOS in the context of AD pathology is crucial for $ONOO^-$ formation. Nevertheless, NO diffusion, which is prominent, can reach non-nitrergic neurons. There is evidence for the involvement of

altered NO production in AD since nNOS has an increased expression in those neurons with neurofibrillary tangles in the entorhinal cortex and hippocampus of AD patients [159]. In addition, increased expression of iNOS and eNOS is associated with neuritic plaques [160,161]. NO production in microglial cells after Aß stimulation has been reported [162], in an effect consisting in iNOS induction in microglial cells following Aß challenge. This suggested the presence of reactive glia surrounding neuritic plaques in AD brains [163,164]. However, the role of microglial iNOS in AD is controversial, as mouse models of AD exhibit neuritic plaques without expressing iNOS neither in microglial nor in astroglial cells [165].

There is experimental evidence that chronic intracerebroventricular perfusion with $Aß_{1-40}$ cause $ONOO^-$ formation and tyrosine nitration [166]. Indeed, specific cerebral regions of AD patients have higher protein nitrotyrosination levels than controls, especially in the hippocampus and the cerebral cortex [167,168], as well as in cerebrospinal fluid (CSF) proteins [169]. The major nitrotyrosinated proteins in AD are related to glucose metabolism (γ-enolase/α-enolase, lactate deshydrogenase and triosephosphate isomerase; TPI) or cellular cytoskeleton (α-actin)[63,170,171]. α-actine and synaptophysin have been also found to be nitrotyrosinated in AD [170]. It has been suggested that the impairment in synapthophysin biological actions might account for the deficit in ACh release characteristic in AD [166]. Interestingly, in mild AD there is a reduction of about 25% in synaptophysin [172]. This reduction could be due to an increase in protein degradation caused by nitrotyrosination.

1.2.7. AD and stroke

Approximately one third of putative cases of vascular dementia have coincidental pathological features of AD. Conversely, ischemic disease

affects 60 to 90% of patients with AD. Therefore, most cases of dementia are in fact mixed. Interestingly, epidemiological reports point towards a causative link between cerebrovascular accident and AD onset. This studies show how the prevalence of dementia in ischemic stroke patients is nine-fold higher than controls 3 months after ischemic stroke [173] and 4 to 12 times higher than in controls 4 years after a lacunar infarct [174]. Direct tissular deterioration by ischemic stroke is responsible of only half of these post-ischemic dementias, suggesting a possible biochemical link between brain ischemia and AD onset. In fact, there are studies demonstrating BACE1 upregulation after ischemic event [175,176], which could be a plausible explanation for relating cerebrovascular accident and amyloidogenesis. Interestingly, a translational arrest after ischemic insult occurs in neurons, in a mechanism involving PERK activation and eIF2α phosphorylation [175,177]. We proposed that, after an ischemic event, PERK induced interference in the eIF2α controlled signalling of BACE1 translation might account for an initial increase in the amyloidogenic cascade, leading to AD onset.

1.3. Stroke

1.3.1. General characteristics

According to the world health organization (WHO) diagnostic criteria, stroke is a neurological deficit, normally focal, that lasts more than 24 hours and which is apparently caused by a vascular compromise. Therefore, stroke induces a brain damage. Unlike myocardial infarction, for example, stroke is not a mechanistically uniform entity. Stroke is an extremely heterogeneous syndrome that can present in a multitude of

clinical manifestations emerging from a variety of underlying pathologies. This makes the stroke epidemiological studies extremely complicated[178].

1.3.2. Types of stroke

There are three main types of stroke: ischemic stroke, intracerebral hemorrhage and subarachnoid hemorrhage. Ischemic stroke is the most common type of stroke, accounting for 80% of the total cases of stroke[179]. It occurs when a brain artery is blocked. If the blockage persists in the time more than a few minutes, brain ischemia will be produced with the subsequent tissue damage and neuronal injury. Several different kinds of diseases can be the cause of ischemic stroke. Atherosclerosis is the major cause of the narrowing of the brain vessels. It will result in the stasis of the blood flow that will eventually lead to thrombus and emboli formation and artery blockade. Another cause of ischemic stroke comes from blood clots in the heart resulting from abnormal heart beating, heart attack or dysfunctions on heart valves. There exist also less frequent causes of ischemic stroke, including the use of drugs, traumatic injury to the blood vessels of the neck or disorders in the blood clotting system.

Ischemic stroke is divided into two main types: thrombotic and embolic. Thrombotic stroke occurs when the cerebral artery becomes blocked by a blood clot that has been formed within the brain. In an embolic stroke, the blood clot that blocks the brain artery was formed somewhere else in the body and travels in the blood stream until finally hit the brain artery.

The second type of stroke is the intracerebral hemorrhage. It is caused by the rupture of an artery within the brain. The blood released out of the vessels results in an increase of pressure within the brain that can severely injury the brain cells. Hypertension is the most common proximal cause accounting for that kind of cerebrovascular accident.

Finally, subarachnoidal hemorrhage is the third type of stroke. It occurs when an artery located at the subarachnoidal space that surrounds the brain is broken. The blood rapidly fills the area resulting in an increase of the pressure with symptoms that can vary from intense headache to consciousness loss or death. These kinds of hemorrhages are caused by abnormal swellings in the arteries at the base of the brain, called cerebral aneurysms. When the swelling is most severe, the blood vessel wall become weak and can rupture.

1.3.3. Tissular considerations

The brain requires a continuous supply of oxygen and glucose to maintain its normal function and viability. The absence of any energetic reservoir tissue within the brain imposes an uninterrupted supply of around 150 g of glucose and 72 L of oxygen per day. Arterial blood brings these nutrients to the brain in a blood flow that has been estimated to be around 800 mL per minute. Neurons are very sensitive to ischemia and their metabolism is almost one hundred per cent aerobic[179]. When the blood supply is interrupted, a dramatic cascade of events takes place within the affected neuronal population. First of all, ATP levels drop far beyond physiological limits and the energy sources are depleted. Consequently, ATP dependent processes such as the activity of the Na^+/K^+ pump are compromised. The failure of the Na^+/K^+ ATPase in the maintenance of the ionic gradients across the plasma membrane is of crucial relevance in cerebral ischemia. The loss of function of the Na^+/K^+ ATPase causes a Na^+, Cl^- and Ca^{+2} influx to the cell and an increase in extracellular K^+, which triggers neuron depolarization and produces the reversal of aminoacid transporters. In such circumstances, voltage-operated and ligand-gated Ca^{+2} channels are recruited and activated increasing free cytosolic Ca^{+2}. The massive

increase in cytosolic Ca^{+2} will enclose a number of deleterious effects. First of all, mitochondria will be overloaded by calcium, something that will drop ATP production by the mitochondrial respiratory chain. Secondly, free cytosolic Ca^{+2} will produce wide cellular damage through the activation of Ca^{+2} dependent phospholipases, proteases and endonucleases. And finally, an excess in cytosolic Ca^{+2} will produce a massive glutamate release through Ca^{+2} dependent exocitosis [180].

The increase of extracellular glutamate is a hallmark of cerebral ischemic injury. Both calcium dependent exocitosis and aminoacids transporter reversal play an important role in the massive glutamate release that takes place after an ischemic episode. Excessive glutamate release will result in excitotoxicity, a pathological process in which AMPA and NMDAR are overactivated leading to Ca^{+2}, Na^{+}, Cl^{-} and H_2O accumulation, cell swelling and cytotoxic edema [180].

Although this biochemical process is considered to be common in all affected neurons, the experimental data supports that different brain centers exhibit differential susceptibility to ischemia. For instance, the striatum is especially sensitive to the ischemic insult. This explains the neurological motor impairments commonly found in most of stroke patients.

Ischemia has a focal impact that targets a specific brain area under the control of the given compromised vascular network. There can be distinguished an ischemic core and a penumbra area, which still has some level of blood perfusion and constitutes the gateway to the abundant infiltration of inflammatory cells into the brain parenchyma. In the ischemic core neurons undergo massive necrotic death, and glutamate is released massively producing excitotoxicity. In the penumbra, neurons are still alive but their survival is compromised in a balance between apoptosis cell death and survival. Therefore, in the penumbra area certain biochemical routes of programmed cell death are activated, opening a

fertile field of research directed to the design of pharmacological tools directed to the neuroprotection of the penumbra area [181].

1.3.4. Oxidative stress in stroke

A large body of experimental research indicates that the generation of free radicals leading to oxidative stress is a main event during ischemia, being a key mediator of the pathophysiological mechanisms underlying ischemic brain damage [182-184]. Although the interruption in the blood flow may be transitory, the tissue damage continues evolving after reperfusion. As we described before, certain population of neurons undergo necrotic death in the ischemic core; nevertheless, in the area where reperfusion occurs, oxidative stress will be generated and tissue damage will start its evolution[185].

Oxidative stress occurs as the result of an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms [186]. Therefore, such stress might occur due to an increase in superoxide ion production ─the common precursor for multiple reactive oxygen and nitrogen species─ or due to a decreased expression or activity of the enzymes whose role consists in controlling ROS levels [187].

The brain parenchyma is particularly vulnerable to the injury induced by ROS. For instance, the lipids found in brain cellular membranes are very rich in polyunsaturated fatty acid side chains, which contain chemical double bonds that are very susceptible to free radicals attack. In addition, the brain contains a low level of antioxidant enzymes such as catalase, SOD and glutathion peroxidase. Therefore, free radicals in the ischemic tissue will provoke damage to lipids, DNA and proteins and will eventually lead to neuronal death [188].

An important goal in order to both understand and fight against oxidative stress consists on the identification of cellular ROS sources. It is known that mitochondria are an important source of superoxide anion, and enzymes with oxidase activity do also have a role in the production of superoxide anion. Recently, there has been much interest regarding the role of NAD(P)H oxidases as potential sources of ROS in cerebral blood vessels since there are evidences of such phenomenon in diabetes and hypertension [189,190]. Hence, the mechanisms that participate in the development of stroke-associated oxidative stress are not completely defined. Further work is needed to evaluate the role of oxidative stress in ischemic stroke.

1.3.5. NO and stroke

Strong experimental evidences point a critical role of NO in the observed neurotoxicity after brain ischemia. This evidences come both from *in vitro* studies in which cell cultures or brain slices are treated with oxygen-glucose deprivation (OGD) and more importantly from *in vivo* models of cerebral ischemia produced by middle cerebral artery occlusion in rodents [191].

As mentioned above, after brain ischemia there is a massive release of glutamate as a consequence of discontrolled Ca^{+2} influx into the cell [180]. Glutamate levels may reach 50 times their normal levels, overexciting ischemic cells [192]. Glutamate antagonists reduce about 50-60% stroke damage [192], supporting a role for glutamate in stroke pathology. Glutamate toxicity associated with stroke can be reproduced in cerebral cortical cultures, where NMDAR activation can kill up to 90% of neurons, whereas NMDAR antagonists prevent this damage [193]. Interestingly, glutamate neurotoxicity is diminished in cultures from nNOS knockout mice or following treatment with NOS inhibitors [194], and treatment with

NOS inhibitors strongly reduces stroke damage [195-197]. This effect is reproduced in nNOS knockout mice [198], which exibit a reduction in stroke-induced damage.

The rationale for the deleterious effects carried out by NO in the context of glutamate toxicity is based in the tight interaction between nNOS and the glutamate receptor NMDAR, which activates the synthesis of NO by NOS through the entry of Ca^{+2}. Therefore, overactivation of NMDAR will result in a burst in NO production due to nNOS activation [199].

However, NO plays a dual role after brain ischemia, being able to exert either protective or deleterious effects. The beneficial or damaging effects of NO will depend on the particular NOS isoform activated, the specific cellular type involved in its generation and the time course of NO production after the brain ischemic injury.

Following brain ischemia, NO produced from eNOS is protective by promoting vasodilatation. Importantly, an increase of the blood flow in the penumbra area is produced in the very early stages of brain ischemia due to the activation of eNOS. This early increase in the blood perfusion will notably improve the survival chances of neuronal populations in the penumbra area [200]. However NO reacts extremely efficiently with superoxide anion resulting in a loss of NO bioavailability. On the other hand, as ischemic injury progresses, NO will be overproduced by the overactivation of nNOS [201-203]. Furthermore, *de novo* synthesis of iNOS will result in an over-release of NO that will contribute to brain damage [204-207].

Experimental work using specific NOS inhibitors and KO mice approaches for nNOS, iNOS and nNOS have clearly established the protective role of eNOS and the damaging role of nNOS and iNOS in the pathophysiological conditions occurring in cerebral ischemia[208-210].

1.3.6 The hemostatic system

The hemostatic system is a paradigmatic physiological system with a very delicate equilibrium. Firstly, circulatory system must be self-sealing. Otherwise, the smallest injury would result in hemorrhages compromising seriously the life. Furthermore, the clotting phenomena that confers to the blood its self-sealing properties must be auto limited. The formation of the clot has to be restricted to the damaged vascular area and the clot must be dissolved when the hurt has been repaired. If not, spontaneous coagulation of the blood (as it occurs *in vitro*) would result in the same harmful consequences as hemorrhage.

Therefore, coagulation is the main defense of the organism against blood loss. The clotting process is initiated by both platelet aggregation and the damaged tissue. Around 20 molecules, most of them glycoproteins synthesized in the liver, take part in this process in which a cascade of proteolytic reactions culminates in the formation of the blood clot or thrombus.

Seven out of the twenty clotting factors are zymogens –inactive forms- of serinproteases that will be in turn activated by other serinproteases placed upstream in the activation cascade. The activation of zymogens to become active serinproteases depends on Ca^{+2} and on a proper phospholipidic environment. Other clotting proteins known as *accessory factors* are also activated by serinproteases and contribute to increase the activation speed of some of the zymogens.

The proteolytic clotting cascade finalizes in the formation of the thrombus, an insoluble matrix that is able to impede blood to leak through. This structure is composed molecularly by inter-crossed fibrin. Fibrin is produced from the soluble plasmatic protein fibrinogen (factor I) through a proteolytic reaction catalysed by the serinprotease thrombin (Fig. 12)

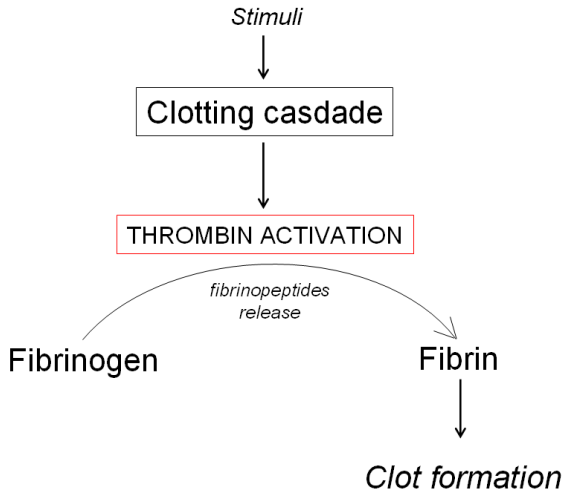

Figure 12. *Finalization of the clotting cascade*

As previously remarked, blood clots are only provisional solutions that must be eliminated as soon as the definite healing is achieved. The elimination process of the blood clot is particularly important when the blood clot has been incorrectly formed or when it has been de-attached from the vessel wall and liberated to the blood flow. To achieve this goal, fibrin is a molecule that has evolved to be easily degraded in a process known as fibrinolysis. The factor that degrades fibrin is named plasmin. Plasmin is a plasmatic serinprotease that specifically cuts fibrin destroying the networks that integrate the blood clot. Plasmin is formed through the proteolytic cut of a zymogen, homolog to the zymogens belonging to the blood clotting cascade, named plasminogen. The serinproteases that proteolyze the plasminogen to convert it into plasmin are known as *plasminogen activators*. Among them, the most relevant physiologically are uroquinase (a serinprotease synthesized in the kidney) and tissular plasminogen activator, present in the vascular tissues. In the complex physiological system coagulation/fibrinolysis there exist some key

molecular players that are prone to be altered by nitrative stress, a kind of stress particularly relevant in stroke.

1.3.7. Fibrinogen

Fibrinogen represents from 2 to 3% of the total plasmatic protein. It is a complex consisting in a heterohexamer whose subunits are linked by disulfide bonds. This complex is formed by two sets of three homolog but non identical polpeptide chains: Aα (610 aminoacids), Bβ (461Aa) and γ (411Aa), and two pairs of oligosacharides linked by N-glycosilation. In this nomenclature, A and B represent the N-terminal fibrinopeptides (with a respective length of 16 and 14 Aa repectively) that thrombin cuts out of the fibrinogen, in such a way that a monomer of fibrin is designated as $\alpha_2\beta_2\gamma_2$. Therefore, the reaction that gives rise to a blood clot formation from fibrinogen can be reduced to the scheme in figure 13.

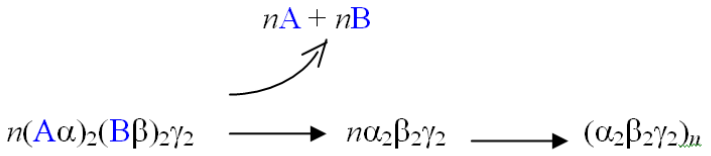

Figure 13. *Fibrinogen conversion to fibrin.* Two fibrinopeptides (A and B) are released from soluble fibrinogen to allow its aggregation to fibrin.

Thrombin specifically cuts the peptidic bond Arg-Gly that links each fibrinopeptide to the fibrin. Immediately after that, fibrin aggregates spontaneously to form fibers. There are two biochemical reasons explaining why fibrinogen, having a structure almost identical to the fibrin can remain in solution whereas fibrin monomers aggregate spontaneously. First of all, the excision of fibrinopeptides facilitates the exhibition of

molecular domains that facilitate intramolecular association. And secondly, fibrinogen aggregation is inhibited by strong electrostatic repulsions as fibrinopeptides contain negatively charged residues.
Finally, fibrinogen, beyond being essential for blood clot formation through its conversion to fibrin, takes part in its soluble form as cofactor in platelet aggregation.

1.3.7.1. FGA

The gen codifying for the fibrinogen Aα chain is named FGA, and by analogy the genes codifying for the Bβ and γ chains and are named FGB and FGG respectively.
FGA gene undergoes alternative splicing generating two distinct isoforms named FGA1 and FGA2 or FGA-ε, with no functional relevance known at the moment. Regarding post-translational modifications, Aα chain can be phosphorylated at several residues.
Abnormalities in the FGA gene can cause a number of pathologic conditions. Congenital afibrinogemia is a rare recessive autosomic disorder that in the most of the cases is originated due to deletions in the FGA gene [211]. The symptoms of this atypical disease are hemorrhages that can be severe in its intensity and periodicity. Mutations in Arg-35, the place where thrombin cuts out fibrinopeptide A, produce alpha-disfibrinogemia. Finally, deficencies in FGA gene are the cause of hereditary kidney amyloidosis [212,213]

1.3.7.2. FGB

There are not neither splicing variants nor post-translational modifications documented for the FGB gene up to the moment. Abnormalities in the

FGB gene that codifies for the Bβ fibrinogen chain are the cause of afibrinogemia congenita (as it occurs with FGA) and thrombophilia [212].

1.3.7.3. FGG

FGG gene encodes the γ chain of the fibrinogen and gives rise to two distinct isoforms through alternative splicing of the transcripts. The two isoforms receive the names Gamma-A and Gamma-B without any functional relevance of the two different isoforms known up to the moment.

Regarding post-translational modifications it is interesting to notice that the γ chain of the fibrinogen undergoes sulfatations in the C-terminal tyrosines in a way to increase its affinity for thrombin [214].

As it occurs with FGA and FGB, abnormalities in FGG originate pathological conditions such as congenital afibrinogemia or thrombophilia [212].

1.3.7.4. Nitrotyrosinated fibrinogen

In a previous work by Vadseth *et al*. it was shown that fibrinogen undergoes nitrotyrosination in coronary artery disease (CAD) patients[215]. Furthermore, in this work relevant changes in the biophysical properties of fibrinogen due to nitrotyrosination are extensively described. Therefore, if the protein complex known as fibrinogen can be nitrotyrosinated in CAD patients, it is a reasonable to speculate that fibrinogen can also be nitrotyrosinated under the nitrative stress conditions occurring in stroke.

Fibrinogen nitrotyrosination implies a series of alterations that produce a pro-thrombotic status, something that can contribute to the worsening of

stroke symptomatology and that could play an important role in the generation of recurrent episodes of stroke. Firstly, nitrotyrosinated fibrinogen exhibits a great acceleration in clot formation (polymerizes faster) when compared with the polymerization kinetics found in control fibrinogen or oxydized fibrinogen. Secondly, fibrin stabilizating factor (FSF or factor XIII) can act in a much more accelerated way in the nitrotyrosinated fibrinogen; this means that FSF will be intercrossing with fibrin with higher affinity than physiologically. Therefore, fibrinolysis is impaired and the uncontrolled growth of blood clots is favored. Finally, the architecture of fibrin networks polymerized from nitrotyrosinated fibrinogen is notably different to the normal architecture that fibrin networks have.

Fibrinogen is a structural protein; therefore, an alteration in its structure affects severely its function. It has been shown that fibrin clots arising from nitrotyrosinated fibrinogen are altered in its viscoelastic properties. This clots composed from nitrotyrosinated fibrinogen are weaker, less stable, more sensitive to the mechanical stresses and can be easily cleaved rising the formation of microemboli [215].

2. Hypothesis and Objectives

2.1. Hypothesis

The present work addresses the study of the physiopathological role of NO in brain. Regarding the physiology of NO, we propose NO as a direct regulator of APP cleavage through the activation of BACE1 translation. This process could be relevant in memory formation as it would regulate the lowering of the adhesion of neuron's terminals to the extracellular matrix, allowing the structural rearrangements required in synaptic plasticity. Regarding the pathological implications of NO when its homeostasis is lost, we propose the NO-derived toxic anion peroxynitrite (formed by the combination of NO and $O_2^{\cdot-}$ in a pro-oxidant environment) as the mediator of most of the cellular damages induced by amyloid ß-peptides. Furthermore, peroxynitrite is a plausible key contributor to stroke-associated damages since brain ischemia is a well-known source of both NO and free radicals.

2.2. Objectives

The main goal of this thesis is the study of the physiological role of NO in brain attending to memory and learning and its pathological role in Alzheimer's disease and Stroke. The partial objectives derived from this main goal are:

2.2.1. **Memory and Learning**: Although BACE1 plays a pivotal role in AD pathogenesis its physiological function in neurons is not understood yet. Uncovering the intracellular signalling that controls BACE1 translation could contribute to elucidate its biological function in the CNS. Then we attempt to study the physiological role of NO in the translational activation of BACE1 through eIF2α phosphorylation in hippocampal neurons.

2.2.2. **Alzheimer's Disease initiation**: There are extraphysiological factors, such as HSV1 infection, that can disrupt eIF2α signalling leading to a non-physiological BACE1 translational activation which will result in BACE1 up-regulation and amyloidogenesis. The study of the role of HSV1 infection in the pathological upregulation of BACE1 through the activation of the eIF2α kinase PKR will be addressed herein.

2.2.3. **Alzheimer's Disease progression**: Aß fibrils in aqueous solution produce H_2O_2. This pro-oxidant agent crosses the plasmatic membranes and damages mitochondria and calcium channels. The production of superoxide anion and NO by H_2O_2 will yield to the formation of peroxynitrite. Then we will study the role of nitro-oxidative stress in Aß-induced cytotoxicity.

2.2.4. **Stroke**: Peroxynitrite could be a central mediator of the brain tissue injures ocurring after brain ischemia. Due to its diffusible nature, peroxynitrite could spread the damage to circulating plasmatic proteins. Fibrinogen is one the most abundant plasmatic protein and a key risk factor in stroke. In this objective we will address the effect that peroxynitrite has in brain tissue damage and in fibrinogen nitrotyrosination after a stroke.

3. Results and Methods

CHAPTER I

"Nitric oxide induces BACE1 translation by Heme-regulated eukaryotic initiation factor(eIF)2α kinase (HRI)-mediated eIF2α phosphorylation"

Gerard ILL-Raga; Francesc X. Guix; Eva Ramos-Fernández; Mònica Bosch; Marta Tajes; César Fandos; Bart de Strooper; Francisco J. Muñoz.

Nitric oxide induces *BACE1* translation by Heme-regulated eukaryotic initiation factor(eIF)2α kinase (HRI)-mediated eIF2α phosphorylation.

Gerard ILL-Raga; Francesc X. Guix; Eva Ramos-Fernández; Mònica Bosch; Marta Tajes; César Fandos; Bart de Strooper; Francisco J. Muñoz.

Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Catalonia, Spain.
Department for developmental and molecular Genetics—VIB, Leuven, Belgium.

Running title*: Nitric oxide controls BACE1 translation*

*To whom correspondence should be addressed:
Dr. Francisco J. Muñoz
Grup de Fisiologia Molecular i Canalopaties
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra
Parc de Recerca Biomèdica de Barcelona,
C/ Dr. Aiguader, 88, Barcelona 08003, Catalonia, Spain
Fax: +34 93 316 09 01; Phone: +34 93 316 08 52
e-mail: paco.munoz@upf.edu

Summary

ß-site APP cleaving enzyme-1 (BACE1) is an aspartyl protease necessary for ß-amyloid production, a crucial event driving AD pathogenesis. However, BACE1 activity is also involved in synaptic plasticity and memory formation. Therefore, BACE1 biology is contradictory. Here, we show that short-lasting stimulation with physiological concentrations of nitric oxide (NO) induces phosphorylation of the eukaryotic initiation factor-2α (eIF2α), which increases the translation of BACE1. Cloning BACE1 5'untranslated region (5'UTR) upstream of a luciferase gene confirmed its inhibitory effect over BACE1 translation, which can be avoided by Salubrinal, an inhibitor of eIF2-P phosphatase PP1c. Treatment of 5'UTR-luc construct with a NO donor mimicked the stimulatory effect exerted by salubrinal over BACE1 translation. Knocking down the expression of the heme-regulated eukaryotic initiation factor(eIF)2α kinase (HRI) blocks the NO induced BACE1 increase. Furthermore, HRI is expressed at synaptic sites, where it colocalizes with BACE1 and phosphorylated eIF2α. These results suggest NO as the neurosignal controlling BACE1 translation. The fact that multiple stress factors commonly present in AD can directly elevate eIF2α phosphorylation levels, disrupting the abovementioned NO signalling pathway, provides a model in wich both BACE1 contradictory faces are harmonized.

Key words: Alzheimer's disease; Nitric Oxide; BACE1; heme-regulated eukaryotic initiation factor-2α kinase; eukaryotic initiation factor-2α

Nonstandard abbreviations

5' untranslated region (5'UTR); Alzheimer's disease (AD); amyloid-ß peptide (Aß); amyloid precursor protein (APP); site Amyloid precursor protein Cleaving Enzyme 1 (BACE1); Dulbecco's modified Eagle's medium (DMEM); eukaryotic initiation factor-2α (eIF2α); heme-regulated eukaryotic initiation factor-2α kinase (HRI); neurofibrilary tangles (NFT); nitric oxide (NO); neuronal NO synthase (nNOS); open reading frame (ORF); [1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one] (ODQ); Post synaptic density protein 95 (PSD95); Salubrinal (Sal003); sodium nitroprusside (SNP); soluble guanylyl cyclase (sGC);

Introduction

Alzheimer's disease (AD) is the most common and devastating dementia among the elderly population. AD is clinically characterized by a progressive decline in the cognitive functions, including loss of memory and loss of the ability to learn and think.

Pathologically, AD is characterized by neuronal loss in different brain regions and by the accumulation of abnormal protein structures in the brain. This abnormal accumulation includes intracellular deposition of neurofibrilary tangles (NFT) and extracellular deposits of various types of amyloid-ß (Aß) in senile plaques.

The amyloid-ß peptide is a polypeptide originated from the proteolytic processing of the amyloid precursor protein (APP), a single pass transmembrane protein with cell adhesion properties. The sequential cleavage of APP by gamma-secretase complex and ß-secretase render the two main Aß isoforms, $Aß_{1-40}$ and $Aß_{1-42.}$ Abnormal Aß accumulation in the brain is considered to be the upstream event driving AD pathogenesis. Nevertheless, the components of the amyloidogenic pathway –those needed for APP processing and Aß generation–, APP, gamma secretase complex and ß-secretase, were identifyed for its implication in the pathology. But, can the amyloidogenic pathway have a physiological relevance? In the present work we address the physiological significance of ß-secretase activity. Beta site Amyloid precursor protein Cleaving Enzyme 1 (BACE1) was identified as the aspartyl protease that cleaves APP in the ß-site in a necessary step to produce the Aß peptide. *BACE1*-KO animal models present no gross abnormalities (*1, 2*). Interestingly, a closer analysis reveals synaptic plasticity deficits and cognitive and emotional behavioural impairments in the *BACE1*-KO mice (*3, 4*). Another study demostrates that BACE1 activity is essential for the APP-mediated enhancement of memory and activity-dependent synaptic

plastictity (*5*). These findings, together with the fact that synaptic activity directly modulates the levels of extracelleular Aß (*6*), led us to hypothesize that a tightly regulated control of BACE1 activity might be crucial for the understanding of the simultaneous implication of BACE1 in synaptic plasticity and amyloid pathology –when this physiological regulation became disrupted by any source of neuronal stress.

We initially observed that administration of physiological concentrations (nanomolar range) of the nitric oxide (NO) donor sodium nitroprusside (SNP) increased BACE1 protein levels in neuroblastoma and hippocampal cells at short times of exposure –less than 1 hour–. NO is a neurotransmitter (*7-10*) with an important role in LTP (*11*). The rapid BACE1 increase in response to NO administration suggested a translation activation from pre-existing *BACE1* mRNA rather than *de novo* transcription of *BACE1* gene. Indeed, *BACE1* translation is minimal under basal conditions due to the repressive effect imposed by its 5' untranslated region (5'UTR) over *BACE1* open reading frame (ORF) (*12-15*). Paradoxically, such translational brake is skipped when global protein synthesis is inhibited as a consequence eIF2α phosphorylation(*16*). Therefore, *BACE1* translation becomes activated when eIF2α undergoes phosphorylation at Serine 51. Interestingly, recent works show that beyond the general role of eIF2α phosphorylation as a modulator of cellular anabolism in response to different kinds of stresses, the synaptic activity-dependent modulation of eIF2α phosphorylation is essential for learning and memory (*17-19*).

At that point, we wondered whether there could be an intracellular mechanism linking NO and *BACE1* translational activation by eIF2α phosphorylation. Interestingly, HRI kinase, an eIF2α kinase, is activated by NO (*20-22*) in the same fashion than soluble guanylyl cyclase (sGC) (*23*). *HRI* has been identified in rabbit reticulocyte lysates (*24, 25*) as one

of the four existing eIF2α kinases. These eIF2α kinases are activated by different stimuli and phosphorylate the alpha subunit of the translation initiation factor 2 alpha at Serine 51 shutting down global protein synthesis (*26*)– but permitting the translation of those mRNAs bearing 5'UTRs with similar features than *BACE1* 5'UTR (>200 nt, rich GC content, upstream AUGs).

For the first time, we report HRI expression in synaptic sites, where we found colocalization of HRI kinase with eIF2α-P and BACE1. Furthermore, in a series of experiments carried out in different neuronal cell models, we demonstrate that BACE1 becomes upregulated in response to NO administration and eIF2α phosphorylation in a pathway that requires HRI kinase activity.

Results

BACE1 upregulation in neuronal cells in response to physiological concentations of NO

Short time stimulation of neuroblastoma cells and hippocampal primary cultures with the NO donor sodium nitroprusside resulted in a marked increase in BACE1 as seen by immunofluorescence (Figure 1A). According to this result, we decided to carry out a dose-response curve stimulating neuroblastoma cells with increasing concentrations of SNP during 30 minutes. Interestingly, the lowest assayed concentration (10 nM) produced a perceptible increase in BACE1 whereas the highest assayed concentration (500 μM) produced no effect in BACE1 expression (Figure 1B). We chose10 nM SNP as working concentration because this is the concentration achieved in physiological conditions when the neuronal NO synthase (nNOS) is active (*27*). We found that stimulating neuroblastoma cells with 10 nM SNP during 40 minutes caused a 2,5 fold increase in BACE1 expression (Figure 1C,D).

Aß production in response to short time stimulation with NO

Next we studied the production of Aß peptides by NO. Since NO caused an increase in BACE1 expression in neuronal cells, it would be expected to increase Aß production aswell. HEK cells overexpressing the Swedish mutation variant of APP were used. These have a double mutation (Lys to Asn at residue 595 plus Met to Leu at position 596), which makes APP more prone to be cleaved by BACE1. Stimulating HEK cells with nanomolar concentrations of SNP for 1 hour we obtained significant increases in both $Aß_{1-40}$ and $Aß_{1-42}$ production (Figure 2).

BACE1 translation activation in response to NO-induced eIF2α phosphorylation

The rapid increase in BACE1 expression and activity in response to NO suggested a translational mechanism rather than *de novo* gene transcription as the underlying cause of the observed effect. BACE1 5'UTR regulates BACE1 translation (*12-15*) enhancing BACE-1 synthesis from its mRNA when eIF2α is phosphorylated at Serine 51 (*16*). Therefore, we cloned BACE1 5'UTR upstream of a luciferase gene in order to carry out reporter assays (Figure 2A). As expected, the presence of BACE1 5'UTR upstream of the luciferase gene strongly repressed the reporter signal (Figure 2B). Salubrinal (Sal003) inhibits the eIF2α phosphatase PP1c (*28*), resulting in a neat increase in eIF2α phosphorylation. We observed that Sal003 treatment was able to increase the reporter signal of 5'UTR-luciferase constructs, further corroborating the positive effect caused by eIF2α phosphorylation over BACE1 5'UTR (Figure 2C). Then we assayed whether short time, physiological concentrations of NO were able to induce eIF2α phosphorylation *in vitro*. We stimulated human neuroblastoma cells with 10 nM SNP during 40

minutes and analysed the levels of eIF2α phosphorylation by western blot (Figure 2D). Interestingly we found a marked increase in eIF2α phosphorylation in the cells that were subjected to SNP treatment. Next, we assayed wether NO could estimulate reporter signal in the 5'UTR-luciferase constructs. We obtained a progressive increase in reporter signal when we treated the cells for 1 hour with increasing concentrations of SNP (Figure 2E). The abovementioned results indicated that eIF2α phosphorylation mediates the stimulatory effect of NO over BACE1 expression.

HRI kinase is essential for BACE1 upregulation in response to NO

HRI kinase is one of the four existing eIF2α kinases but the only one whose activity is activated by NO in the same fashion than soluble guanylyl cyclase (*20-22, 29-31*). We wanted to test whether HRI kinase activity was the mechanistical link between NO and BACE1 expression through eIF2α phosphorylation. For this we used [1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one] (ODQ), a chemical compound that oxidizes heme groups. We expected that the chemical inactivation of the heme groups present in HRI kinase would abrogate the response of the kinase to NO. As seen in figure 4A, neuroblastoma cells preincubated with ODQ fail to upregulate BACE1 in response to SNP. As ODQ is broadly used to inhibit the sGC pathway, we treated the cells with dbcGMP and observed no positive effect on BACE1 expression (Figure 4B). In a further step we knocked down HRI expression in neuroblastoma cells using an small interferring RNA against HRI (si HRI). Again, we demonstated how the cells lacking HRI kinase failed to upregulate BACE1 in response to SNP (Figure 4C).

The NO/HRI/p-eIF2a pathway is active in synaptosomes

To asses the physiological relevance of BACE1 translational activation in response to NO in the context of neurotransmitter signalling, we addressed the study of the NO/HRI/p-eIF2α pathway in synaptosomal preparations. Interestingly, we found that stimulation of synaptomsomes with physiological concentrations of NO induced BACE1 protein up-regulation and a notable increase in eIF2α phosphorylation levels (Fig. 5A,B). Importantly, HRI kinase is found in synaptosomes (Fig. 6B). This results indicate that the NO-induced BACE1 up-regulation is independent of the neuronal soma and of any event of gene transcriptional activation.

BACE1, HRI kinase and phospphorylated eIF2α are localized at synaptic sites of cultured hippocampal neurons

HRI kinase has been described in reticulocytes (*24, 25*) altough it is known to be expressed in the hippocampus and in the amygdala. We wanted to know whether HRI kinase was expressed at synaptic sites, where it could behave as a molecular sensor for the NO released from the nNOS. Hippocampal primary cultures were analysed for HRI, BACE1 and p-eI2Fα localization by immunofluorescence (Figure 6A). Post synaptic density protein 95 (PSD95) was used as a marker of synaptic sites. Interestingly, PSD95 physically interacts with nNOS, therefore highlighting sites of potential NO production. As shown in panel 5C, BACE1, HRI and p-eIF2α colocalize with PSD95 in the dendritic terminations of hippocampals cultures.

Experimental procedures

Cell cultures

Human neuroblastoma cells (SHSY5Y) were grown with F-12 (Ham) supplemented with 15% fetal bovine serum (FBS) and antibiotics (100

units/mL penicillin and 100 mg/mL streptomycin). HEK cells overexpressing the Swedish mutation of APP and HeLa cells were grown with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Mouse embryo hippocampal cell cultures

Hippocampal 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. Hippocampi were aseptically dissected and trypsinized. Cells were seeded in phenol-red-free DMEM plus 10% horse serum into 1% poly-L-Lysine coated coverslips ($5x10^4$ cells/cover). After 120 min, medium was removed and neurobasal medium was added containing 1% B27 supplement (Gibco BRL) plus antibiotics. Cultured hippocampal cells were used for the
experiments on day 7 when they are considered to be mature neurons (*32*).

Treatments

For the study of BACE1 upregulation in response to sodium nitroprusside dihydrate treatment (SNP, Sigma), neuronal cells were seeded at 65% confluency. Solid SNP was diluted at the appropiate concentration in complete F-12 (Ham) growing medium. After cells were subjected to short time stimulation with SNP, they were immediately either harvested for WB analysis, fixed with 4% PFA for immunofluorescence, lysed for luciferase activity determination or the medium was collected for Aß quantification.

To study the effect of eIF2α phosphatases inhibition over BACE1 5'UTR, HeLa cells were transfected with the 5'UTR-luc construct and subsequently incubated with 100 μM Sal003 for 24h before luciferase activity was determined. To inhibit prostetic heme groups, SH-SY5Y cells were pre-incubated with [1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one] (ODQ) 1 μM for 30 minutes before treatment with SNP.

Protein identification by Western Blot

Cells were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiotreitol, pH 7.4 and a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by Bio-Rad protein assay. For endogenous BACE1 analysis, 80 μg of protein were loaded into the SDS-PAGE for elecrophoretic separation. Protein samples were electrophoretically resolved within 10% Tris-HCl gels ran at 150 V for 1 h and afterwards transferred to nitrocellulose membranes using iBlot Gel Transfer System (Invitrogen). Membranes were blocked in Tween 20-Tris buffer solution (TTBS: 100 mM Tris-HCl, 150 mM NaCl, pH 7.5), containing 5% milk and incubated overnight at 4ºC with 1:1000 rabbit anti-BACE1 (Chemicon International), 1:500 rabbit anti phospho-eIF2α (Ser51) (Cell signalling), 1:1000 mouse anti-eIF2α (Cell signalling) and 1:10,000 mouse anti-tubulin (Sigma) respectively. Primary Abs were diluted either in 5% skimmed milk-TTBS (anti-BACE1 and anti-tubulin) or in 5% bovine serum albumin in TTBS (anti-phospho-eIF2α and anti-eIF2α). Peroxidase-conjugated donkey anti-rabbit and anti-mouse Abs (Amersham Bioscience, Buckinghamshire, U.K.) were used as secondary Abs at 1:5000 for 1 h at RT. Bands were visualized with Super Signal (Pierce, Rockford, IL, USA) and Amersham Bioscience Hyperfilm ECL

kit. Optical density analysis of the bands was carried out by ImageJ software (http://rsbweb.nih.gov/ij/).

Immunofluorescence assays

Human neuroblastoma SH-SY5Y cells ($4x10^4$ cells/cover) were seeded on 1.5% gelatine coated 12 mm coverslips and kept in normal growyh medium. Mouse hippocampal cells ($5x10^4$ cells/cover) were seeded into 1% poly-L-Lysine coated coverslips and kept in neurobasal medium containing 1% B27 supplement (Gibco BRL) plus antibiotics. Immediately after treatment with SNP, cells were fixed with 4% paraformaldehyde (PFA). Afterwards, cells were permeabilized with 0.1% Triton X-100 and subsequently incubated for 2 h at RT in a hydration chamber with 1:100 rabbit anti BACE1 Ab (Chemicon International), 1:500 rabbit anti HRI (Upstate), 1:100 rabbit anti phospho-eIF2α (Serine 51) (Abcam) or 1:200 mouse anti PSD95 (Abcam) respectively. After primary antibody incubation, cells were incubated with 1:700 Alexa Fluor 555 goat anti-rabbit Ab and/or 1:700 Alexa Fluor 488 goat anti-mouse Ab for 1 h at RT. Finally, nuclei were stained with 1:100 TO-PRO 3 iodide (Sigma) in PBS for 10 minutes before mounting. After coverslips were mounted digital images were taken with a Leica TCS SP confocal µscope and analysed with Leica confocal software (Heidelberg, Germany). For colocalization experiments of BACE1, HRI and p-eIF2α with PSD95, each primary antibody was tested with its counter secondary antibody to discard any cross reactivity between rabbit and mouse isotypes.

Aß measurement

HEK cells overexpressing APPsw were grown in DMEM + 10% FBS and antibiotics. Prior to SNP treatment, the growth medium was replaced for experimental medium: phenol red free DMEM containing 4,5 g/L D-glucose, L-glutamine, HEPES (25mM), antibiotics (100 units/mL

penicillin and 10-6 mg/mL streptomycin) and supplemented with 0,2% FBS. After 1h of SNP treatment, 800 ml of medium were collected and centrifuged at 13,000 rpm for 5 minutes to eliminate cellular debris. Solid-phase sandwich ELISA kits containing two highly specific Ab for detection of the Ab peptides were used following manufacturer's instructions to measure human Ab (1–40) and (1–42) (IBL Codes). Briefly, samples were added to ELISA plates pre-coated with anti-human Ab (35–40) mouse IgG affinity purified monoclonal Ab or anti-human Ab(38–42) rabbit IgG affinity purified Ab. After overnight incubation and washing, labelled Ab solution (horseradish peroxidase-conjugated anti-human Ab (N) rabbit IgG affinity purified Ab or horseradish peroxidase-conjugated antihuman Ab (N) rabbit IgG Fab affinity purified) was added for 1 h at RT and then washed. The chromagen (tetramethyl benzidine solution) was added and incubated for 30 min. The absorbance at 450 nm was determined per each
sample.

Cloning of BACE1 5'-untranslated region

Total RNA was extracted from SH-SY5Y cells, and one-step RT-PCR was carried out using kit (Qiagen) with primers designed to amplify BACE1 5'UTR: 5'-GAAGCTTACAAGTCTTTCCGCCTCCCC- 3', 5'-GAAGCTTGGTGGGCCCCGGCCTTC- 3'. PCR product, a single band matching the molecular weight of BACE1 5'UTR (~500 nt), was isolated and purified from an agarose gel using the Ilustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Helthcare) 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 from Promega containing the CMV promoter cloned at BglII and HindIII sites).

Transient DNA transfection of HeLa cells and Luciferase assay

HeLa cells were seeded in 96-well plates at a density of 9,000 cells per well and grown for 12 h with DMEM plus 10% FBS. 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 + 25 ng of CMV-Luciferase Vector + 200 ng of pcDNA3 as controls, and finally 25 ng of Renilla + 25 ng of BACE1-5'UTR CMV-Luciferase construct + 200 ng of pcDNA3 as problem samples. Cells were transfected using JetPEI transfection reagent (PolyPlus) for 2h. Afterwards, medium was changed and cells were incubated for 10 h to allow sufficient gene expression. After treaatments with Sal003 or SNP, cells were lysed and luciferase and Renila activities were
measured by using the Dual-Glo™ Luciferase Assay System (Promega) following manufacturer's instructions and luminescence was read using a luminometer (Fluostar OPTIMA, BMG labtech).

HRI expression knock-down

Human neuroblastoma SH-SY5Y cells ($4x10^4$ cells/cover) were seeded on 1.5% gelatine coated 12 mm coverslips at 90% confluency and transfected to 20 pmoles of HRI siRNA (5'-SI00105784 -3') (Qiagen) or control siRNA (5'-AATTCTCCGAACGTGTCACGT-3') (Qiagen) diluted into 100 µl serum-free medium for 48h. Cells were transfected by a Lipofectamine 2000 (Invitrogen) procedure following the manufacturer's instructions, as described previously (*33*).

Preparation of cortical synaptosomes

Cortical synaptosomes were obtained as described elsewhere (Myhre and Fonnum, 2001, with minor modifications). Two mice were decapitated, their brains were rapidly removed, and the cortex were dissected and homogenized in cold homogenization buffer (5 mM Tris–HCl and 320

mM sucrose), using a borosilicate glass homogenizing tube fitted with a Teflon pestle and filtered through two layers of surgical gauze. The homogenate was centrifuged at 1000 × *g* at 4 °C for 10 min. The supernatant 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 supernatant 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 to a final protein concentration of about 0.1 mg/ml. Protein concentration was determined using Bio-Rad protein reagent. The final synaptosomes suspension was distributed in 1 ml aliquots to perform the experiments. Synaptosomes integrity was assessed by electron microscopy.

Discussion

Our data indicates that physiological concentrations of nitric oxide induce BACE1 expression and activity in a mechanism that involves eIF2α phosphorylation at Serine 51. HRI kinase is the molecular sensor that undergoes activation upon nitric oxide binding (*20-22*), catalysing the phosphorylation of eIF2α at S51 and, as a consequence of that, triggering rapid *BACE1* translation initiation. Importantly, all of the four elements of this pathway, nNOS, NO, HRI, eIF2α and BACE1, are present at synaptic density sites.

According to these findings, we propose a role for nitric oxide as a neurotransmitter that signals to eIF2α being a fine-tune physiological regulator of BACE1 activity. Interestingly, a recent work suggests that the NO pathway is involved in hippocampal Aß production (*34*). However,

apparently these findings do not fit with the stablished molecular basis of AD neurodegeneration in which *BACE1* plays a negative role by triggering Aß production.

Central to AD is progressive memory deteroration. Recent works by Costa-Mattioli and colleagues have demostrated the importance of eIF2α phosphorylation at Serine 51 for synaptic plasticity (*17-19*), learning and memory. They found that the synaptic activity-dependent fluctuation in eIF2α phosphorylation is a crucial event for LTP and memory formation (*17-19*). The latter statement implies that any non-physiological interference in eIF2α phosphorylation levels at synaptic sites can have detrimental effects for synaptic plasticity and memory formation. Importantly, many cellular stresses such as ER stress, glucose deprivation, viral infection and oxidative stress cause eIF2α phosphorylation through activation of specific eIF2α kinases (Figure 7) because the primary role of eIF2α phosphorylation is to arrest global protein synthesis uneder circumstances of cellular compromise. Nonetheless, in the compartimentalized environment of a dendritic spine a transient global arrest in protein synthesis promoted by eIF2α phosphorylation has not major consequences in the global cellular metabolism and can constitute a mean of activating the synthesis of specific genes with special features in its 5'UTRs such as *BACE1*. Indeed, translational regulation of messengers is an important event in highly polarized cells such as neurons are (*35*). This translational control of gene expression is particulary relevant in the understanding of synaptic plasticity (*35-38*), and the nature of the signals (neurotransmitters, neurotrophins) that couple to the translational machinery is a focus of current research (*39-41*).

We speculate that the tightly controlled cleavage of APP at synaptic sites enables a relative deattachment of the dendritic spines from the extracelular matrix. This partial deattachment allows neuronal

terminations to undergo the prominent estructural reorganizations required for neuronal plasticity. Interestingly, APP has been linked to memory-related processes(*42*). We postulate nitric oxide as the neurosignal that regulates APP cleavage and dendrite reorganization by controlling BACE1 activity at the translational level. According to this hypothesis, AD causing mutations in presenilins and APP alter APP metabolism interferring with plasticity processes involving dendrite rearrangement. Aß overproduction, tough important for its neurotoxic effects, sould be considered as an epiphenomenon and not the primary cause of memory deterioration. In this regard, down's syndromes harbour an extra copy of APP gene and they invariably develop AD. It is feasible to speculate that in these individuals, dendritic terminations carrying an extra load of APP have enhanced attachment to the extracellular matrix and, as a consequence of that, a higher resitance to undergo deatachment and reorganization resulting in a decreased neuronal plasticity. However, further work is nedeed to evaluate the plausability of this hypothesis.

A current effort is being carried out to develop BACE1 inhibitors to amiliorate AD pathology by lowering Aß levels (*43*). Many preventions must be taken in this kind of aproaches, as BACE1 activity is required for normal synaptic plasticity (*3-5*). However, lowering BACE1 global activity in a scenario such as AD (in which BACE1 is overexpressed) could be benefitial. Nevertheless, a more convenient therapeutical approach is to adress the primary causes of BACE1 dysregulation, as they are intimately linked with the memory formation processes that might be disrupted in AD.

Our work provides a bridge that joins the two contradictory sides of *BACE1* biology: its positive role in synaptic plasticity and its negative role in promoting neurotoxic Aß production. BACE1 activity is not detrimental for neurons, on the contrary, its activity might be benefitial – when it is controlled by nitric oxide– by promoting dendritic rearrangment

and neuronal plasticity. What is detrimental for neurons is the loss of control over BACE1 tightly regulated expression. Such loss of control over BACE1 expression occurs when multiple stress factors concomitant with AD (Figure 7) induce eIF2α phosphorylation disturbing *BACE1* delicate translation control at synaptic sites. In the future it will be interesting to study the contribution of these AD-related stresses to eIF2α phosphorylation attending to BACE1 dysregulation. In addition, it will be challenging to discover other downstream genes whose translation is regulated by nitric oxide, HRI kinase and eIF2α phosphorylation in the same fashion than BACE1 is.

FIGURE 1

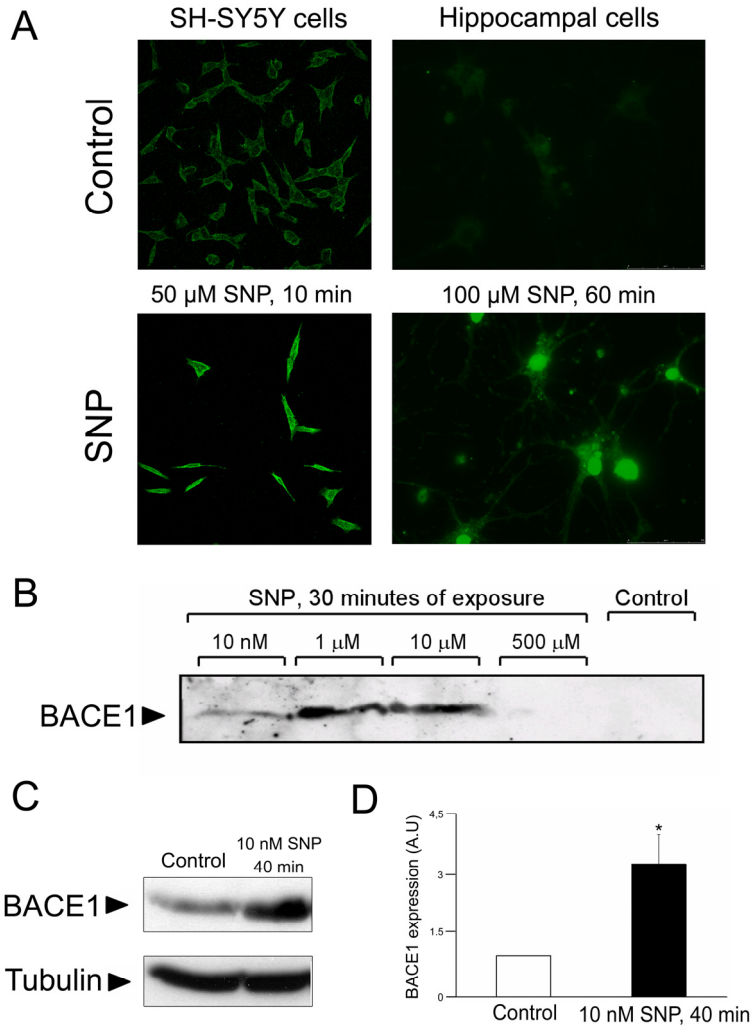

Figure 1 BACE1 is upregulated in neuronal cells in response to physiological concentations of nitric oxide. Human neuroblastoma cells (SH-SY5Y) and mouse hippocampal neurons were exposed to the nitric oxide donor SNP at 50 µM (50 minutes) or 100 µM (60 minutes) respectively and BACE1 expression was analyzed by immunofluorescence (**A**). A dose-response curve was carried out in SH-SY5Y cells with increasing SNP concentrations at a fixed exposure time of 30 minutes (**B**). BACE1 protein up-regulation in response to 40 minutes 10 nM SNP stimulation was assesed by western blot (**C**). Optical density values of BACE1 protein bands were normalized against tubulin (**D**). Data are the mean ± SEM values of four independent experiments; *$p<0.05$.

FIGURE 2

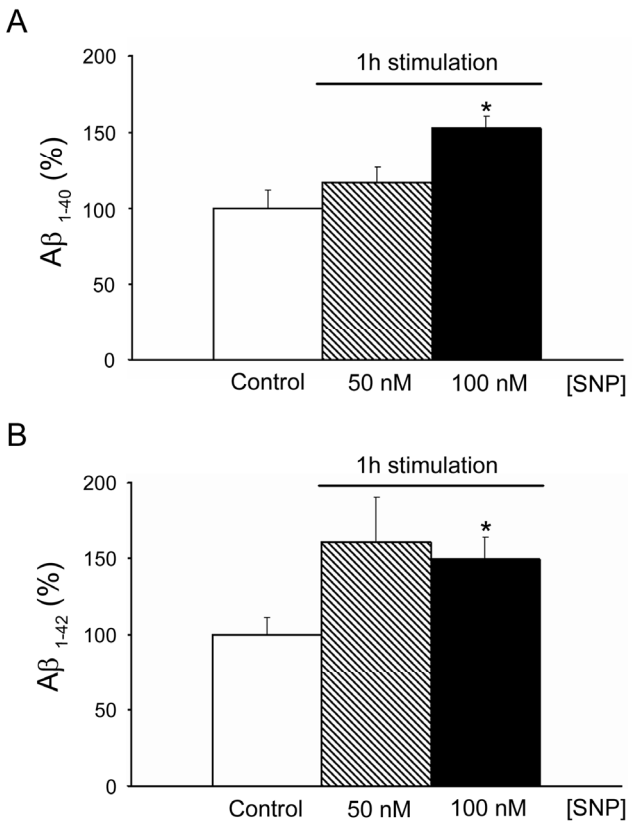

Figure 2 Aß production in response to short time stimulation with nitric oxide. Production of $Aß_{1-40}$ (**A**) and $Aß_{1-42}$ (**B**) was quantified in HEK-APPsw cells in response to 60 minutes stimulation with nanomolar concentrations SNP.

FIGURE 3

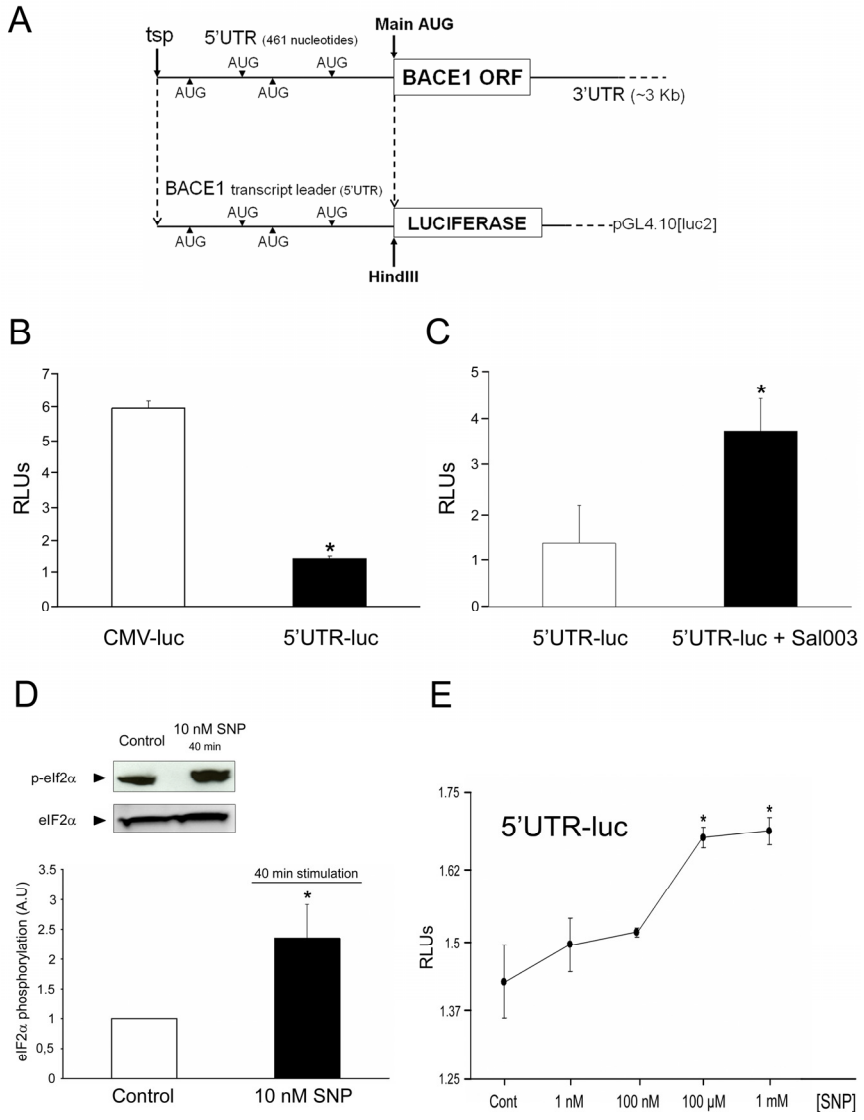

Figure 3 BACE1 translation activation in response to nitric oxide-induced eIF2α phosphorylation. 5' untranslated region (5'UTR) of *BACE1* was cloned and inserted upstream of a luciferase reporter gene (**A**). **B**, Presence of BACE1 5'UTR strongly downregulates luciferase signal. *$p<0.0001$. **C**, Sal003 increases reporter signal in *luciferase* constructs under the control of BACE1 5'UTR. *$p<0.05$. **D**, SH-SY5Y cells were stimulated with 10 nM SNP for 40 minutes and eIF2α phosphorylation levels were analyzed by western blot.*$p<0.05$. **E,** SH-SY5Y cells were transfected with the 5'UTR-luc contruct and stimulated with SNP.

FIGURE 4

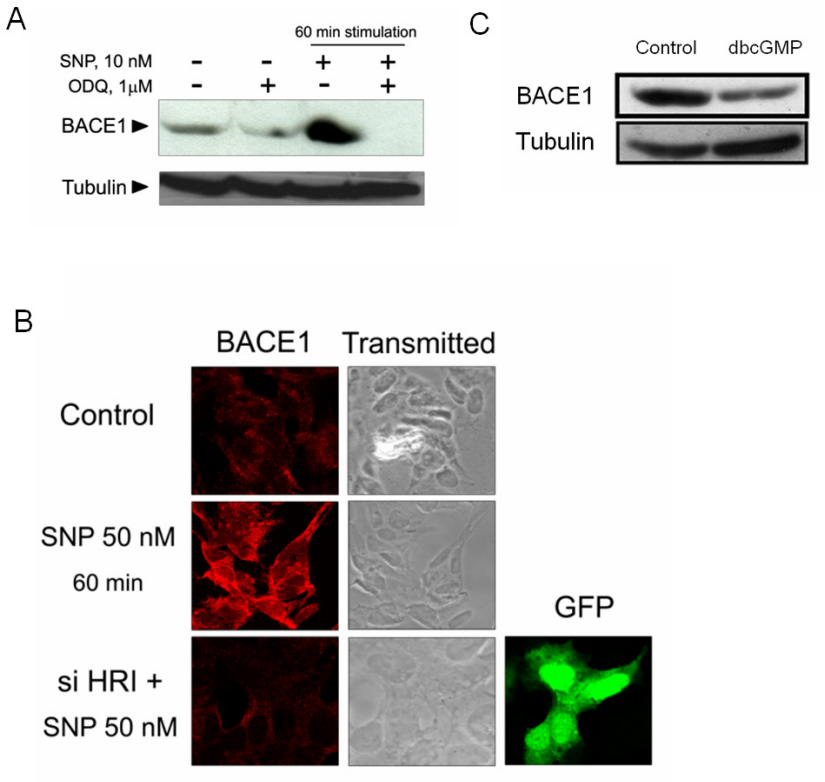

Figure 4 HRI kinase is essential for BACE1 upregulation in response to nitric oxide. **A**. Pre-incubation of SH-SY5Y cells with 1 μM ODQ for 30 minutes was carried out in order to achieve complete heme group inhibition before assaying 10 nM SNP stimulation in ODQ-treated and control cells. SNP stimulation lasted 1h. BACE1 protein levels were determined by western blot. Tubuline was used as loading control. **B**. The sGC pathway is not involved in the BACE1 expression up-regulation induced by NO. SH-SY5Y cells were treated with the cGMP analog dbcGMP (500 μM) for 40 minutes and no BACE1 up-regulation was observed. **C**. HRI kinase was knocked down in SH-SY5Y cells by transfecting them with siHRI RNA during 48h. BACE1 expression in response to SNP treatment (50nM, 1h) was assayed by immunofluorescence comparing resting cells with siHRI transfected cells. GFP was used to monitor the efficency of siRNA transfection.

FIGURE 5

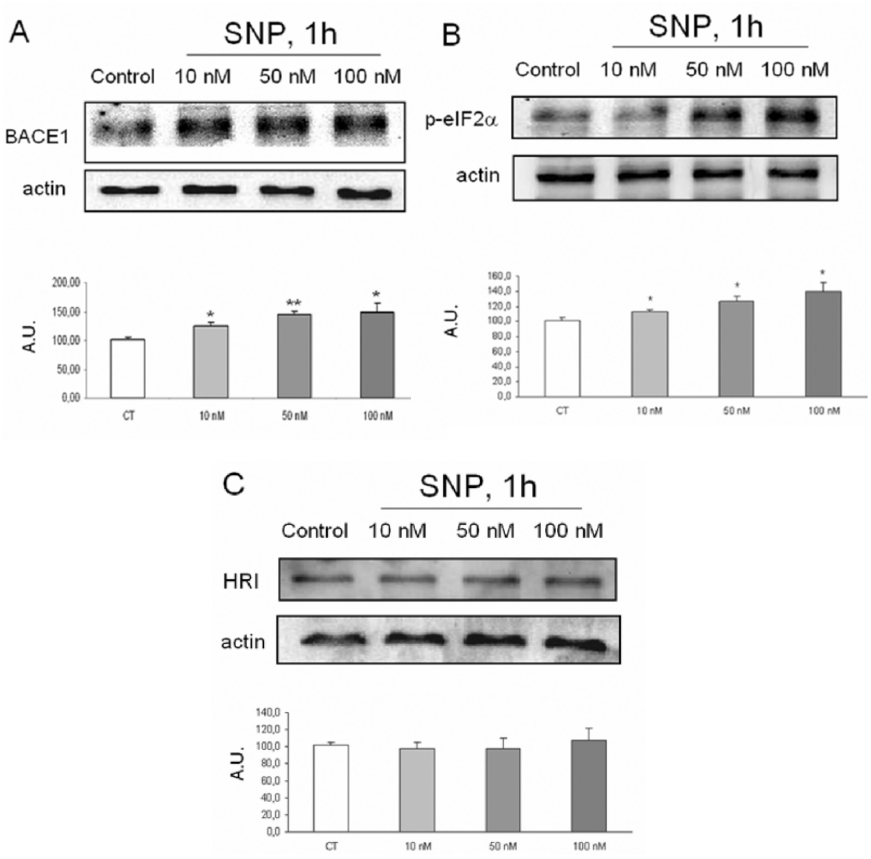

Figure 5 Nitric oxide induces the expression of BACE1 in synaptosomes. Synaptosomes were treated with physiological concentrations of NO (10, 50 and 100 nM) for 1 h. BACE1 and p-eIF2α levels were increased in a dose dependent manner (**A,B**). Importantly, HRI kinase is present in synaptosomes (**C**).

FIGURE 6

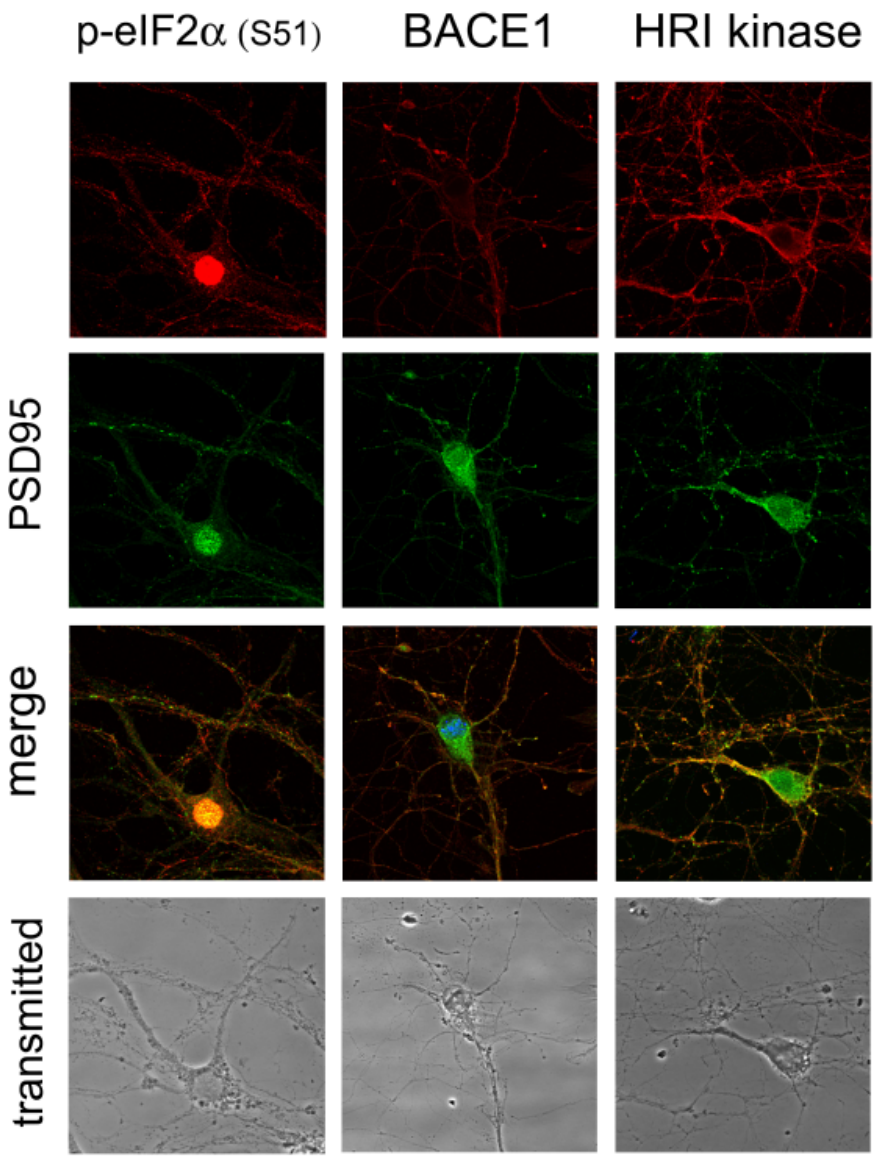

Figure 6. BACE1, HRI kinase and p-eIF2α are localized at synaptic sites. Cultured mouse hippocampal cells were used to study the cellular distribution of BACE1, HRI kinase and p-eIF2α. Post synaptic density protein 95 (PSD95) was used a synaptic sites marker.

FIGURE 7

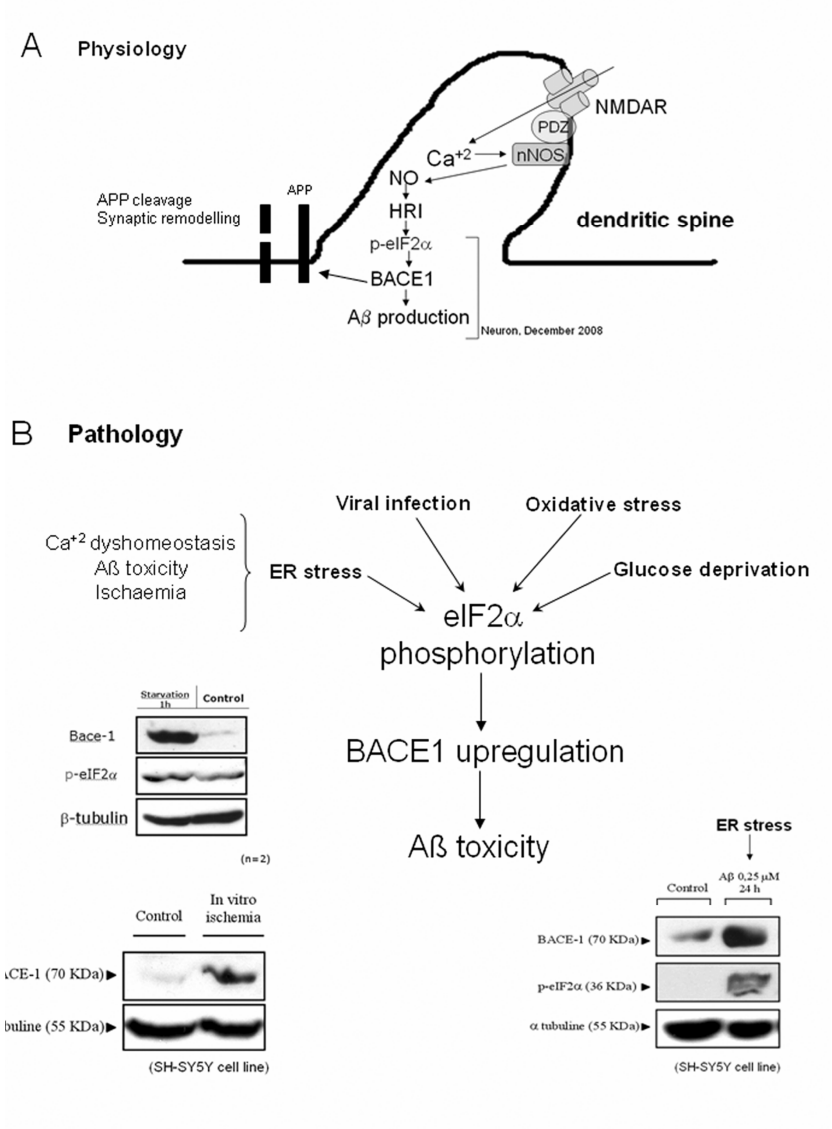

Figure 7. A, Signalling pathway controlling BACE1 translation. **B**, Stress factors disrupt eIF2α phosphorylation pathway, leading to discontrolled BACE1 overexpression and Aß production.

References

1. Y. Luo *et al.*, *Nat. Neurosci.* **4**, 231 (2001).
2. S. L. Roberds *et al.*, *Hum. Mol. Genet.* **10**, 1317 (2001).
3. F. M. Laird *et al.*, *J. Neurosci.* **25**, 11693 (2005).
4. H. Wang, L. Song, F. Laird, P. C. Wong, H. K. Lee, *J. Neurosci.* **28**, 8677 (2008).
5. H. Ma *et al.*, *Proc. Natl. Acad. Sci. U. S. A* **104**, 8167 (2007).
6. J. R. Cirrito *et al.*, *Neuron* **48**, 913 (2005).
7. H. Bult *et al.*, *Nature* **345**, 346 (1990).
8. G. E. Boeckxstaens *et al.*, *Eur. J. Pharmacol.* **190**, 239 (1990).
9. R. Zakhary *et al.*, *Proc. Natl. Acad. Sci. U. S. A* **94**, 14848 (1997).
10. L. Xue *et al.*, *Proc. Natl. Acad. Sci. U. S. A* **97**, 1851 (2000).
11. H. Son *et al.*, *Cell* **87**, 1015 (1996).
12. T. D. De Pietri *et al.*, *Nucleic Acids Res.* **32**, 1808 (2004).
13. S. Lammich, S. Schobel, A. K. Zimmer, S. F. Lichtenthaler, C. Haass, *EMBO Rep.* **5**, 620 (2004).
14. W. Zhou, W. Song, *Mol. Cell Biol.* **26**, 3353 (2006).
15. M. Mihailovich, R. Thermann, F. Grohovaz, M. W. Hentze, D. Zacchetti, *Nucleic Acids Res.* **35**, 2975 (2007).
16. T. O'Connor *et al.*, *Neuron* **60**, 988 (2008).
17. M. Costa-Mattioli, N. Sonenberg, *Crit Rev. Neurobiol.* **18**, 187 (2006).
18. M. Costa-Mattioli *et al.*, *Nature* **436**, 1166 (2005).
19. M. Costa-Mattioli *et al.*, *Cell* **129**, 195 (2007).

20. S. Uma, B. G. Yun, R. L. Matts, *J. Biol. Chem.* **276**, 14875 (2001).

21. H. Ishikawa *et al.*, *J. Am. Chem. Soc.* **124**, 13696 (2002).

22. J. Igarashi *et al.*, *J. Biol. Chem.* **279**, 15752 (2004).

23. L. J. Ignarro, *Blood Vessels* **28**, 67 (1991).

24. J. J. Chen. Translational regulation in reticulocytes: the role of heme-regulated eIF-2alpha kinase. 349-372. 1993. New York, Plenum Place.

25. J. J. Chen, I. M. London, *Trends Biochem. Sci.* **20**, 105 (1995).

26. H. C. de, R. Mendez, J. Santoyo, *FASEB J.* **10**, 1378 (1996).

27. F. X. Guix, I. Uribesalgo, M. Coma, F. J. Munoz, *Prog. Neurobiol.* **76**, 126 (2005).

28. M. Boyce *et al.*, *Science* **307**, 935 (2005).

29. M. Rafie-Kolpin *et al.*, *J. Biol. Chem.* **275**, 5171 (2000).

30. S. Uma, R. L. Matts, Y. Guo, S. White, J. J. Chen, *Eur. J. Biochem.* **267**, 498 (2000).

31. M. Miksanova *et al.*, *Biochemistry* **45**, 9894 (2006).

32. S. Kaech, G. Banker, *Nat. Protoc.* **1**, 2406 (2006).

33. M. Arniges, E. Vazquez, J. M. Fernandez-Fernandez, M. A. Valverde, *J. Biol. Chem.* **279**, 54062 (2004).

34. S. Li, W. Wang, C. Wang, Y. Y. Tang, *Neurosci. Lett.* (2009).

35. S. Kindler, H. Wang, D. Richter, H. Tiedge, *Annu. Rev. Cell Dev. Biol.* **21**, 223 (2005).

36. M. A. Sutton, E. M. Schuman, *Cell* **127**, 49 (2006).

37. M. A. Sutton, E. M. Schuman, *J. Neurobiol.* **64**, 116 (2005).

38. E. M. Schuman, *Neuron* **23**, 645 (1999).

39. E. Klann, M. D. Antion, J. L. Banko, L. Hou, *Learn. Mem.* **11**, 365 (2004).

40. E. Klann, T. E. Dever, *Nat. Rev. Neurosci.* **5**, 931 (2004).

41. C. R. Bramham, D. G. Wells, *Nat. Rev. Neurosci.* **8**, 776 (2007).

42. G. R. Dawson *et al.*, *Neuroscience* **90**, 1 (1999).

43. C. E. Hunt, A. J. Turner, *FEBS J.* **276**, 1845 (2009).

CHAPTER II

"Herpes Simplex Virus 1 upregulates BACE1 by PKR activation"

Gerard ILL-Raga, Ernest Palomer, Matthew A. Wozniak, Eva Ramos, Francesc X. Guix, José J.Galán, Jordi Clarimón, Carmen Antúnez, Luis M. Real, Mercé Boada, Ruth F. Itzhaki, César Fandos, and Francisco J. Muñoz

Herpes Simplex Virus 1 upregulates BACE1 by PKR activation

Gerard ILL-Raga[a], Ernest Palomer[a], Matthew A. Wozniak[b], Eva Ramos[a], Francesc X. Guix[a], José J.Galán[c], Jordi Clarimón[d], Carmen Antúnez[e], Luis M. Real[c], Mercé Boada[f,g], Ruth F. Itzhaki[b], César Fandos[a], and Francisco J. Muñoz[a *]

[a]Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Catalonia, Spain; [b]University of Manchester, UK; [c]NeoCodex, Sevilla, Spain; [d]Alzheimer Laboratory, Neurology Department, Hospital de la Santa Creu i Sant Pau, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Barcelona; [e]Fundación Alzheimur, Unidad de Demencias, Hospital Virgen de la Arrixaca de Murcia; [f]Memory Clinic of ACE Foundation. Catalan Institute of Applied Neurosciences, Barcelona; [g]Neurology Department, Hospital G. Universitari Vall d'Hebron, Barcelona.

Running title: *HSV1 activates BACE1 translation*

[*]To whom correspondence should be addressed:
Dr. Francisco J. Muñoz
Grup de Fisiologia Molecular i Canalopaties
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra
C/ Dr. Aiguader, 88, Barcelona 08003, Catalonia, Spain
Fax: +34 93 316 09 01; Phone: +34 93 316 08 52
e-mail: paco.munoz@upf.edu

Abstract

BACE1 is necessary for amyloid ß-peptide (Aß) production in Alzheimer's disease (AD). Here we show that herpes simplex virus type-1 (HSV1) upregulates BACE1. As we demonstrate in HSV1-infected neuroblastoma cells and in peripheral nervous tissue from HSV1-infected mouse, HSV1 activates PKR. PKR is an eukaryotic initiation factor (eIF)2α kinase activated during viral genome replication by double stranded RNA (dsRNA). eIF2α phosphorylation reverses the inhibitory effect exerted by BACE1 5'untranslated region (5'UTR) over BACE1 translation. Cloning BACE1 5'UTR upstream of a luciferase (luc) gene confirmed its inhibitory effect, which can be avoided by salubrinal, an inhibitor of the eIF2α phosphatase PP1c. Treatment with the dsRNA analog poly (I:C) mimicked the stimulatory effect exerted by salubrinal over BACE1 translation in the 5'UTR-luc construct and increased Aß production in HEK-APPsw cells. Finally activated PKR was found only in the AD brain tissue and not in the aged-matched control. A genetic association case-control analysis revealed that the rs2254985 polymorphism within the PKR gene showed a trend towards Alzheimer's disease risk (gender and APOE-adjusted OR for the TT genotype = 1.24, 95% CI: 1.00-1.53, P = 0.05).

Keywords: Alzheimer's disease; PKR; BACE1; amyloid; HSV1.

Abbreviations: Alzheimer disease (AD); amyloid β-protein (Aβ); amyloid precursor protein (APP); apolipoprotein E gene (APOE-ε4); β-site APP Cleaving Enzyme (BACE1); Cytomegalovirus (CMV); double stranded RNA (dsRNA); double-stranded RNA-activated protein kinase (PKR); eukaryotic initiation factor 2-α (eIF2α); Eukaryotic translationinitiation

factor 2-alpha kinase 2 (EIF2AK2); general control non-derepressible 2 kinase (GCN2); HEK cells overexpressing the Swedish mutation of APP (HEK-APPsw); hemeregulated inhibitor kinase (HRI); herpes simplex encephalitis (HSE); herpes simplex virus type 1 (HSV1); luciferase (luc); open reading frame (ORF); phosphorylated eIF2α (peIF2α); PKR-like ER-localized eIF2α kinase (PERK); polyinosinic–polycytidylic acid [poly(I:C)]; Salubrinal (Sal003); Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PPP1CA); Single nucleotide polymorphism (SNP); 5'untranslated region (5'UTR); upstream initiation codons (uAUGs).

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that affects 18 million people worldwide. Sufferers experience severe memory deficits and cognitive decline, and their brains are characterized by two pathological features – neurofibrillary tangles and senile plaques. The former consist mainly of abnormally phosphorylated forms of the microtubule associated protein tau, and the latter of amyloid β-protein (Aβ) (Glenner and Wong, 1984), a peptide generated by the enzymatic cleavage of an integral membrane glycoprotein called amyloid precursor protein (APP). One of the enzymes involved in APP cleavage is BACE1 (β-site APP Cleaving Enzyme) (Vassar et al., 1999; Sinha et al., 1999; Hussain et al., 1999; Yan et al., 1999; Lin et al., 2000), a single pass transmembrane peptidase belonging to the aspartyl protease family (Del Toro et al., 2009). BACE1 expression is elevated in AD patients by unclear causes (Marcinkiewicz and Seidah, 2000; Gatta et al., 2002; Preece et al., 2003) . Interestingly, BACE1 protein levels are elevated in AD brains whereas BACE1 mRNA levels remain unaltered (Gatta et al., 2002; Preece et al., 2003; Marcinkiewicz and Seidah, 2000). Furthermore, despite the fact that stimuli such as oxidative stress (Tamagno et al., 2002; Coma et al., 2008) or hypoxia (Sun et al., 2006; Zhang et al., 2007) activate BACE1 transcription, upregulating BACE1 mRNA levels, BACE1 translation is basally repressed by its 5'untranslated region (5'UTR) (De Pietri et al., 2004; Lammich et al., 2004; Zhou and Song, 2006; Mihailovich et al., 2007). The 5'UTR of BACE1 is 470 nucleotides long, rich in GC (~70%) and it contains four upstream initiation codons (uAUGs). All four uAUGs, but especially the second one, are responsible for the translational arrest that the 5'UTR exerts over the BACE1 open reading frame (ORF) (Lammich et al., 2004).

The eukaryotic initiation factor 2-α (eIF2α) is a GTP-binding protein that catalyses the loading of the first met-tRNA into the ribosome to initiate protein translation (Morris and Geballe, 2000). When eIF2α is phosphorylated at Ser51, translation initiation is arrested, a scenario required in the event of cellular compromise such as glucose deprivation, ER stress or viral infection (Wek et al., 2006). However, there is a specific subset of mRNAs whose translation is activated in response to eIF2α phosphorylation (Hinnebusch, 1996; Hinnebusch, 1997). A recent study shows that BACE1 belongs to this subset of mRNAs demonstrating that eIF2α phosphorylation activates BACE1 translation (O'Connor et al., 2008). Consequently, factors that promote phosphorylation of eIF2α at Ser51 might contribute to elevated BACE1 expression, leading to an increase in cleavage of APP and Aβ accumulation.

There are four kinases that are capable of phosphorylating eIF2α at Ser51: doublestranded RNA-activated protein kinase (PKR), PKR-like ER-localized eIF2α kinase (PERK), heme-regulated inhibitor kinase (HRI; present in erythrocytes) and general control nonderepressible 2 kinase (GCN2; activated by amino acid deprivation) (De Haro et al., 1996). PKR has an N-terminal domain that behaves as a molecular sensor for any double stranded RNA (dsRNA) formed during the replication of viral genomes (Taylor et al., 2005). Molecular recognition of dsRNA at the PKR N-terminal domain induces its autophosphorylation at multiple sites but especially at Thr446, which activates the kinase, catalysing eIF2α phosphorylation at Ser51. Therefore, PKR is a defensive viral sensor that shuts off global protein synthesis to prevent viral protein replication within the host cell (Williams, 1999). However, this defensive cellular mechanism might have a negative side effect, as BACE1 translation is activated in response to eIF2α phosphorylation (O'Connor et al., 2008), causing breakdown of APP to Aβ. Thus PKR activation might

be an important factor in AD, and in fact, a potentially functional variation of EIF2AK2 gene (the gene coding for PKR) has been associated with AD (Bullido et al., 2008) and activated PKR has been found in the brains of AD sufferers (Peel and Bredesen, 2003).

Many viruses cause PKR activation but only one of these has been consistently linked to AD – herpes simplex virus type 1 (HSV1). HSV1 is a neurotropic virus that infects most humans, usually during infancy. Once a person is infected, the virus persists lifelong in the peripheral nervous system. HSV1 is the cause of several diseases including herpes labialis, genital herpes and a severe, but rare, brain disorder called herpes simplex encephalitis (HSE). HSV1 was originally proposed as a factor in AD because the brain regions that are affected in HSE are the same as those in AD – the frontal and temporal cortices (Ball, 1982). Subsequent work has shown that HSV1 is present in the brains of elderly humans (Jamieson et al., 1991) and that it confers risk of AD in subjects who possess a specific genetic factor: the type 4 allele of the apolipoprotein E gene (APOE-ε4) (Itzhaki et al., 1997). Recently, the virus was shown to cause Aβ accumulation (Wozniak et al., 2007) and abnormal tau phosphorylation (Wozniak et al., 2009a), and to increase the levels of the associated enzymes, including BACE1 (Wozniak et al., 2007). Also, HSV1 DNA was found to be very specifically associated with amyloid plaques in AD brains (Wozniak et al., 2009b).

The mechanism by which HSV1 induces BACE1 expression is unknown. However, it is a plausible hypothesis that PKR activation and subsequent eIF2α phosphorylation activate BACE1 translation in HSV1-infected neurons. In the present work we investigated this possibility. Firstly, we present evidence that HSV1 infection activates PKR in neuroblastoma cells and in dorsal root ganglion (DRG) from HSV1-infected mice. To provide a mechanistic insight into the way PKR induces BACE1 translation, we cloned BACE1 5'UTR and inserted it upstream of a

luciferase (luc) gene. As expected, BACE1 5'UTR repressed the reporter signal. Salubrinal (Sal003), an inhibitor of the eIF2α phosphatase PP1c, derepressed the reporter signal in the 5'UTR-luc construct, demonstrating a positive effect exerted by eIF2α phosphorylation over the 5'UTR-luc construct. Synthetic polyinosinic–polycytidylic acid (poly [I:C]), which is an analog of dsRNA (Alexopoulou et al., 2001; Matsumoto et al., 2004), resulted in *in vitro* PKR activation and produced a recovery in 5'UTR-luc reporter signal comparable to that obtained by Sal003. Furthermore, a specific PKR inhibitor (Jammi et al., 2003) reversed the rise in reporter signal induced by poly (I:C), thereby indicating that PKR triggers BACE1 translation.

Previous studies reported PKR activation in neurons from AD brains (Chang et al., 2002b; Peel and Bredesen, 2003). We confirmed these findings in brain sections that were previously tested for HSV1 infection (Wozniak et al., 2009b). Interestingly, activated PKR was only present in AD neurones although HSV1 DNA was present in the tissue from a nondemented subject as well. This result led us to analyse the AD-association of two polymorphisms (rs2254985 and 7480390) in two genes, EIF2AK2 (PKR) and PPP1CA (phosphatase-1 catalytic subunit), whose differential interaction with HSV1 proteins might help to explain why PKR is selectively activated in AD sufferers.

2. Materials and Methods

2.1. Reagents

Synthetic dsRNA poly (I:C) was purchased from Sigma-Aldrich (St. Louis, MO, USA). An imidazolo-oxindole compound that acts as a potent ATP-binding site directed inhibitor of PKR (Jammi et al., 2003) was purchased from Calbiochem (San Diego, CA, USA). All media and

additives for cell culture were purchased from Gibco (Carlsbad, CA, USA).

2.2. Cell cultures

Human neuroblastoma cells (SH-SY5Y) were grown with F-12 (Ham) supplemented with 15% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). HEK cells overexpressing the Swedish mutation of APP (HEK-APPsw) were kindly provided by Dr. B. de Strooper (K.U. Leuven, Belgium) and HeLa cells were grown with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2.

2.3. Mouse embryo hippocampal cell cultures

Hippocampal cells were isolated from 18-day-old CB1 mouse embryos. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Hippocampi were aseptically dissected and trypsinized. Cells were seeded in phenol-red-free DMEM plus 10% horse serum on to 1% poly-L-Lysine coated coverslips ($5x10^4$ cells/cover). After 120 min, medium was removed and neurobasal medium was added containing 1% B27 supplement (Gibco BRL) plus antibiotics. Cultured hippocampal cells were used for the experiments on day 7 when they were considered to be mature neurons (Kaech and Banker, 2006).

2.4. Human brain samples

Serial temporal lobe sections from an AD patient and an age-matched control were kindly provided by the South West Dementia Brain Bank, Bristol, UK. The individuals analysed were a 78 years old control female

carrying the APOE genotype E3E3, and a 75 years old AD female carrying the APOE genotype E3E4. A previous study (Wozniak et al., 2009b) carried out on sequential sections of the same samples revealed that HSV1 DNA was present in both.

2.5. Mouse dorsal ganglion samples

Sections of DRG from HSV1-infected mice were kindly provided by Dr Stacey Efstathiou, University of Cambridge, UK. Five 8-9 week old BALB/c mice were infected with 106 plaque forming units (pfu) of HSV1 strain SC16 by ear scarification in the left ear pinna. Five days after infection the animals were killed and the CII, CIII and CIV cervical DRG were pooled from the five mice. The contralateral sensory ganglia from the same animals were used as control, uninfected ganglia.

2.6. Protein identification by Western Blot

Cells were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiotreitol, pH 7.4 and a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by Bio-Rad protein assay. Protein samples were electrophoretically resolved within 10% Tris-HCl gels ran at 150 V for 1 h and afterwards transferred to nitrocellulose membranes using iBlot Gel Transfer System (Invitrogen). Membranes were blocked in Tween 20-Tris buffer solution (TTBS: 0.1% v/v Tween 20, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5), containing 5% milk, and incubated overnight at 4ºC with 1:1000 rabbit anti-PKR (phosphoT446; Abcam), 1:500 rabbit anti Phospho-eIF2α (Ser51; p-eIF2α) (Cell signalling), 1:1000 rabbit anti-BACE1 (Chemicon International) and 1:1000 mouse antieIF2α (Cell signalling), respectively. Primary antibodies were diluted either in 5% skimmed milk-TTBS (anti-phosphoPKR and anti-BACE1) or in 5% bovine serum albumin in TTBS

(anti-p-eIF2α and anti-eIF2α). Peroxidase-conjugated donkey anti-rabbit and anti-mouse (Amersham Bioscience, Buckinghamshire, U.K.) were used as secondary Abs at 1:5000 for 1h at RT. Bands were visualized with Super Signal (Pierce, Rockford, IL, USA) and Amersham Bioscience Hyperfilm ECL kit.

2.7. Infection of SH-SY5Y cells with HSV1

HSV1 (strain SC16) stocks were prepared as described previously (Dobson et al., 2006). SH-SY5Y cells were seeded at a concentration of 10.74 million cells per flask (T75) and incubated overnight. Prior to infection, growth medium was discarded and cells were then washed briefly with 10 mL of PBS at 37°C. HSV1 was added to 1 mL of 0.5% FBS supplemented growth medium at 3 pfu/cell. During the 1 h of incubation, the HSV1-inoculated medium was gently distributed homogeneously on the flask surface. This procedure was repeated four times, every 15 min. Afterwards, the inoculated medium was removed, and 10 mL of fresh growth medium plus 0.5% FBS was added, followed by incubation for 24 h. For luciferase assays of BACE1 5'UTR, cells could not be infected with HSV1, as they do not resist subsequent infection and transfection procedures as we have obtained in our lab. For that purpose, we used poly (I:C) as an accepted model for viral infection (Alexopoulou et al., 2001; Matsumoto et al., 2004; Scheuner et al., 2006).

2.8. Immunocytochemistry, immunohistochemstry and immunofluorescence

For immunocytochemistry, SH-SY5Y cells were grown on aminopropylethoxysilane (APES)-coated glass slides and infected with HSV1 (one pfu)/cell). Slides were fixed for 10 min in 4% formalin and 10% acetic acid in PBS, washed and left overnight in 70% ethanol. Afterwards, slides were washed twice, for 5 min, in Tris-Buffer saline

(TBS) and then rinsed in 20% (v/v) acetic acid for 45 s to inhibit endogenous alkaline phosphatase activity. Next, slides were washed twice in TBS containing 0.025% (v/v) Triton X-100 and blocked in 10% (w/v) skimmed milk in TBS for 1h at RT on a shaker. After two further washes in TBS, primary antibodies were applied in 1% (w/v) skimmed milk in TBS and incubated overnight in an hydration chamber at the following dilutions: 1:100 rabbit anti p-eIF2α (Ser51) (Cell signalling), 1:100 rabbit anti-PKR and 1:100 rabbit anti BACE1 (Abcam). Slides were rinsed in TBS containing 0.025% Triton X-100 and treated for 2 h with a biotinylated secondary Ab (Abcam) diluted in TBS plus 1% skimmed milk. Slides were again washed twice in TBS and then treated with streptavidin-alkaline phosphatase conjugate (Sigma) for 30 min. After two further washes in TBS, substrate (5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium) was added and slides incubated for 30–120 min before rinsing, mounting and UV curing. For immunohistochemistry, human brain temporal lobe sections and mouse DRG sections were dehydrated by treating successively with 70%, 90% and absolute ethanol, dewaxed in 100% xylene and rehydrated by using the ethanol series in reverse. Next, slides were rinsed three times in PBS for five minutes per wash, placed in an acidic buffer (sodium citrate 10 mM, pH 6) and autoclaved for one cycle in a Prestige Medical. Afterwards, slides were rinsed 3 times in TBS and the procedure continued as described above from the blocking of endogenous alkaline phosphatase activity step. For immunofluorescence, hippocampal primary cultures were fixed with 4% paraformaldehyde (PFA) after treatment with poly (I:C) at a final concentration of 5μg/mL for 2.5 h. Afterwards, cells were permeabilized with 0.1% Triton X-100 and subsequently incubated for 2 h at RT in a hydration chamber with 1:100 rabbit anti BACE1 Ab (Chemicon International), followed by incubation with 1:700 Alexa Fluor 488 goat anti-rabbit polyclonal Ab for 1 h at RT. Finally, nuclei were

stained with TO-PRO 3 iodide (Sigma). Coverslips were mounted and digital images were taken with a Leica TCS SP confocal microscope and analysed with Leica confocal software (Heidelberg, Germany).

2.9. Cloning of BACE1 5'-untranslated region

Total RNA was extracted from SH-SY5Y cells, and one-step RT-PCR was carried out using a kit (Qiagen) with primers designed to amplify BACE1 5'UTR: 5'-GAAGCTTACAAGTCTTTCCGCCTCCCC-3', 5'-GAAGCTTGGTGGGCCCCGGCCTTC-3'. PCR product, a single band matching the molecular weight of BACE1 5'UTR (~500 nt), was isolated and purified from an agarose gel using the Ilustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) 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 from Promega containing the CMV promoter cloned at BglII and HindIII sites).

2.10. Transient DNA transfection of HeLa cells and Luciferase assay

HeLa cells were seeded in 96-well plates at a density of 9000 cells per well and grown for 12 h with DMEM plus 10% FBS. 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 + 25 ng of CMV-Luciferase Vector + 200 ng of pcDNA3 as controls, and finally 25 ng of Renilla + 25 ng of BACE1-5'UTR CMV-Luciferase construct + 200 ng of pcDNA3 as test samples. Cells were transfected using JetPEI transfection reagent (PolyPlus) for 2h. Afterwards, medium was changed and cells were incubated for 10h to allow sufficient gene expression. Then, the wells were preincubated with PKR inhibitor (Calbiochem) in DMEM supplemented with 10 % FBS at a final concentration of 0.3 μM for 1h. Next, medium containing PKR inhibitor was withdrawn, cells were washed once with PBS and replaced

with DMEM plus 1% FBS, with or without polyinosinic:polycytidylic acid (poly (I:C); Sigma) at a final concentration of 5 μg/mL for 3h. Luciferase and Renila activities were measured by using the Dual-Glo™ Luciferase Assay System (Promega) following the manufacturer's instructions, and luminescence was read using a luminometer (Fluostar OPTIMA, BMG labtech).

2.11. Aß measurement

HEK-APPsw were grown and the growth medium was replaced for experimental medium: phenol-red free DMEM containing 4.5 g/L D-glucose, L-glutamine, HEPES (25mM), antibiotics (100 units/mL penicillin and 10-6 μg/mL streptomycin) and supplemented with 0.2% FBS. 800 μl of medium was collected in each experimental condition, and centrifuged at 13,000 rpm for 5 minutes to eliminate cellular debris. Solidphase sandwich ELISA kits containing two highly specific Ab for detection of the Aβ peptides were used following manufacturer's instructions to measure human Aβ(1–40) and (1–42) (IBL Codes 27714 and 27712 respectively). Briefly, samples were added to ELISA plates pre-coated with anti-human Aβ(35–40) mouse IgG affinity purified monoclonal Ab or anti-human Aβ(38–42) rabbit IgG affinity purified Ab. After overnight incubation and washing, labelled Ab solution (horseradish peroxidase-conjugated anti-human Aβ (N) rabbit IgG affinity purified Ab or horseradish peroxidase-conjugated anti-human Aβ (N) rabbit IgGFab affinity purified) was added for 1 h at RT and then washed. The chromagen (tetramethyl benzidine solution) was added and incubated for 30 min. The absorbance at 450 nm was determined per each sample.

2.12. AD and control individual selection

This study is part of an on-going multi-center study on the genetic basis of AD in Spain. Blood samples are being collected from consecutive AD patients in medical centers in Barcelona, Madrid and Murcia. The referral centers' ethics committees and Neocodex have approved this research protocol, which was in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association. Written informed consents were obtained from all individuals included in this study. This analysis comprised 1122 unrelated sporadic AD patients (mean age at diagnosis ± SD: 77.55 ± 7.73 years old) and 1191 unrelated controls (52.14 ± 11.95). Control subjects were recruited from the general population. All AD patients fulfilled DSM-IV criteria for dementia and were diagnosed according to the NINCDS-ADRDA criteria for possible and probable AD (McKhann et al., 1984). All patients received a thorough clinical and neurological examination and a comprehensive neuropsychological evaluation including tests for general cognition, memory, language, perceptual and constructional abilities and executive functions. Complete blood analysis and neuroimaging studies were performed in all subjects to exclude other potential causes of dementia. Following the guidelines for the diagnosis of AD from the Study group on Behavioral Neurology and Dementia of the Spanish Neurological Society, AD patients were consecutively recruited at the four participating centers: Fundació ACE-Institut Català de Neurociències Aplicades, Barcelona; Hospital Universitario La Paz-Cantoblanco (Madrid, Spain); and Unidad de Demencias, Hospital Virgen de la Arrixaca and Fundación Alzheimur (Murcia, Spain). To avoid population stratification problems, all individuals enrolled in this study were white Mediterranean with registered Spanish ancestors (two generations), as recorded by clinical researchers.

2.13. DNA extraction procedures

We obtained 5 mL of peripheral blood from all individuals to isolate genomic DNA from leukocytes. DNA extraction was performed in a MagNa Pure LC Instrument (Roche, Basel, Switzerland), using MagNa Pure LC DNA Isolation kit (Roche) in accordance with the manufacturer's instructions.

2.14. Genetic association study

Genotypes for the *EIF2AK2* rs2254958 polymorphism, which is located at the 5'-UTR region within a putative exonic splicing enhancer, were obtained using Real-time PCR. Primers and probes employed for this genotyping protocol are summarized in Table 1. The technique was performed in the LightCycler ® 480 System (Roche Diagnostics). Briefly, PCR was carried out in a final volume of 20 μL using 10 ng of genomic DNA, 0.1 μM of forward amplification primer, 0.25 μM of reverse amplification primer, 0.1 μM each detection probe, and 4 μL of LC480 Genotyping Master (5X, Roche Diagnostics, Germany). We used an initial denaturation step of 95ºC for 5 min, followed by 50 cycles of 95ºC for 30 sec, 62ºC for 30 sec, and 72ºC for 30 sec. For melting curve analyses, after an initial denaturation at 95ºC for 2 minutes at a ramp rate of 4.4ºC/second, the samples were incubated at 64ºC for 30 sec and 40ºC for 30 sec at a ramp rate of 2.2ºC/sec, and finally taken to 80ºC with one acquisition per ºC. APOE genotyping protocols have been previously described (Ramirez et al., 2009). We explored the association of rs2254958 markers with AD phenotype using different tests adapted from Sasieni based on chi-squared test (http://ihg2.helmholtzmuenchen.de/). SPSS statistical package was used to develop binary logistic regression models adjusting for age, gender and *APOE* dosage.

3. Results

3.1. HSV1 infection activates PKR

Previous research has shown that infection with HSV1 leads to activation of PKR which, in turn, causes phosphorylation of eIF2α and consequently triggers a shut-off of global protein synthesis (Chou et al., 1995). We confirmed this finding in neuroblastoma cells (SH-SY5Y) infected with HSV1 for 24h (Figs. 1A and 1B). Immunocytochemistry analysis showed a striking rise in PKR activation (phosphorylated at Thr446) in HSV1-infected cells (Fig. 1A, upper panels) that was accompanied by a rise in the signal corresponding to p-eIF2α (middle panels) and BACE1 (lower panels). Western blot analysis of HSV1-infected neuroblastoma cells revealed a prominent band for activated PKR that was absent in the control uninfected cells (Fig. 1B). Western blotting confirmed that peIF2α levels are larger in HSV1-infected than in uninfected cells. Non-phosphorylated eIF2α was used as load control demonstrating that total expression of eIF2α was not affected by the virus. To further study PKR activation by HSV1, we analysed peripheral nervous tissue from mice infected with HSV1. Consistently with the results obtained in neuroblastoma cells, DRG infected with HSV1 showed a striking activation of PKR whereas no signal for active PKR appeared on the contralateral uninfected DRG.

Activated PKR phosphorylates eIF2α at Ser51, enhancing BACE1 translation (O'Connor et al., 2008). Thus, HSV1 infection might be predicted to cause an increase in BACE1 protein via PKR and eIF2α phosphorylation. Indeed, previous work has shown that BACE1 is increased in HSV1-infected cells (Wozniak et al., 2007).

3.2. Poly (I:C) induces PKR activation, BACE1 upregulation and Aß production in cultured cells.

We used poly (I:C), a synthetic viral dsRNA (Alexopoulou et al., 2001; Matsumoto et al., 2004; Scheuner et al., 2006), to promote PKR activation *in vitro.* As shown in Fig. 2A, poly (I:C) treatment (5 μg/mL) induces the active form of PKR (phosphorylated at Thr446) in HeLa cells. Interestingly, BACE1 protein becomes rapidly upregulated in response to the initial rise of p-PKR(Thr446) obtained after only 1h of stimulation with poly (I:C) (Fig. 2A). The same effect occurs in primary hippocampal cultures, in which high expression of BACE1, localized in the neurite network of hippocampal neurons, is observed after brief (2h) incubation with poly (I:C) (Fig. 2B).

The prominent BACE1 up regulation in response to the viral dsRNA mimetic poly (I:C) (Figs. 2A and 2B) led us to ask whether poly (I:C) could also trigger Aß production *in vitro*. We found that poly (I:C) triggers both Aß1-40 and Aß1-42 production (Figs. 2C and 2D) in HEK-APPsw cells. HEK-APPsw cells have a double mutation (Lys to Asn at residue 595 plus Met to Leu at position 596), which makes APP more prone to cleavage by BACE1, thus producing early AD onset (Citron et al., 1992). HEK-APPsw treated with poly (I:C) showed a dramatic increase in Aβ1-40 (approximately to 15-fold of the control) after 3h ($p<0.005$) (Fig. 2C). After 6h the level of Aβ1-40 decreased to 7-fold above the control but still remained significantly higher ($p<0.005$) than the untreated value. The pattern for Aβ1-42 (Fig. 2D) was similar to that obtained with Aβ1-40. Thus 3h after poly (I:C) treatment, Aβ1-42 increases to 2 fold of the control value ($p<0.05$); after 6h Aβ1-42 levels decrease slightly but are still higher than control levels.

3.3- BACE1 translation is activated in a mechanism dependent on PKR activation and eIF2α
phosphorylation.

BACE1 translation is inhibited under basal conditions by its 5'UTR (De Pietri et al., 2004; Lammich et al., 2004; Zhou and Song, 2006; Mihailovich et al., 2007), but this is reversed in response to eIF2α phosphorylation (ser51) (O'Connor et al., 2008). We cloned BACE1 5'UTR and inserted it upstream of a reporter luciferase gene (5'UTR-luc) (Fig. 3A). We used this 5'UTR-luc construct for transfection of HeLa cells. As expected, the basal effect of BACE1 5'UTR was to repress translation (Fig. 3B). The high reporter signal obtained with a strong cytomegalovirus promoter (CMV-luc) is reduced about three-fold when BACE1 5'UTR is inserted upstream of the luciferase gene (5'UTR-luc) without deleting the CMV promoter (Fig. 3B). Sal003, an inhibitor of eIF2α phosphatase PP1c, derepressed the reporter signal in 5'UTR-luc constructs, indicating that eIF2α phosphorylation activates BACE1 translation, overcoming the repressor effect imposed by its 5'UTR (Fig.3C). Interestingly, poly (I:C) treatment resulted in the same effect as that obtained with Sal003 (Fig. 3D), lifting the repression of the 5'UTR-luc reporter signal with a detectable increase in luciferase signal. Importantly, this increase in luciferase signal obtained with poly (I:C) treatment was fully reversed when cells were previously incubated with a specific imidazolo-oxindole PKR inhibitor (Jammi et al., 2003) (Fig. 3D).

3.4. PKR activation in human brains

Previous studies have documented PKR activation in AD brains (Chang et al., 2002b; Peel and Bredesen, 2003). To confirm these findings, we chose brain sections from an AD patient and a non-demented individual both of which were infected with HSV1 (Wozniak et al., 2009b). Interestingly, PKR activation was only detected in the AD brain tissue (Fig. 4). HSV1 has its own mechanisms to circumvent host defensive mechanisms. One of these viral strategies consists of avoiding PKR activation by binding

dsRNA species with viral proteins (Cassady and Gross, 2002). However, this system would be working deficiently in AD individuals, who would present a striking activation of PKR. To investigate this, we examined host gene polymorphisms that might be linked to AD through their interaction with HSV1-mediated PKR-circumvention mechanisms.

3.5. EIF2AK2 rs2254985 polymorphism and AD susceptibility

Our data strongly suggest a biological link between the PKR protein and the HSV1 activation of the amyloidogenic APP pathway. Interestingly, a biallelic polymorphism (rs2254985) located in a highly conserved SRp55 binding enhancer element of the *EIF2AK2* gene (which encodes PKR protein) has recently been associated with AD age of onset and susceptibility to AD (Bullido et al., 2008). We carried out an independent case-control genetic association study in order to validate these original findings, but found that rs2254985 allele and genotype frequencies were not statistically different between AD patients and controls (Table 2). In order to address whether the APOE-ε4 allele could be a confounding factor in our analyses, we used the Odds ratio homogeneity test but, in contrast to Bullido et al. (2008), we did not detect any interaction between APOE-ε4 and rs2254985 (p=0.580). However, we observed a trend to association of the rs2254985 TT genotype (recessive model) with AD susceptibility when we carried out a binary logistic regression analysis adjusting for gender and APOE (Table 3). Finally, we performed a Cox regression analysis to test the effect of the rs2254985 TT genotype polymorphism on the age of AD onset, adjusting for APOE and gender, but no statistically significant results were obtained.

4. Discussion

In the present work we provide experimental data supporting the proposition that HSV1 is a major risk factor for AD (Itzhaki et al., 1997; Bullido et al., 2008; Wozniak et al., 2007), acting perhaps by increasing the Aβ level via an increased expression of BACE1 (Wozniak et al., 2007). We suggest that the BACE increase occurs through activation of PKR, leading to BACE1 up-regulation through eIF2α phosphorylation-mediated BACE1 translational de-repression. The p-eIF2α inhibition of translation affects most mRNAs except those bearing a 5'UTR with specific features: long (> 200 nucleotides), rich in GC content (~70%) and containing upstream initiation codons; these mRNAs, including BACE1 mRNA, will be more efficiently translated into proteins during infection as a consequence of eIF2α phosphorylation. Therefore, the increase in BACE1 expression characteristic of AD could be partially due to the translational derepression over BACE1 mRNA exerted by HSV1. However, when the translational arrest in global protein synthesis is sustained over time, the infected cell undergoes programmed cell death (Lee and Esteban, 1994; Der et al., 1997; Tan and Katze, 1999). Interestingly, in recent years an involvement of PKR in neurodegeneration including AD has been reported (Chang et al., 2002a), which proposes a proapoptotic role for activated PKR (Peel and Bredesen, 2003; Chang et al., 2002b; Peel, 2004). Intermittent activation of PKR during the lifetime of neurons by transient activation of latent HSV1 could contribute to increase APP amyloidogenic cleavage. Other causes of BACE1 activation by eIF2α phosphorylation have been investigated. A recent study (O'Connor et al., 2008) showed that cellular stress induced by glucose deprivation leads to BACE1 translation via eIF2α phosphorylation, in an effect mediated by PERK, one of the four kinases that are able to phosphorylate eIF2α. (De Haro et al., 1996). Indeed, we have found that poly (I:C), a viral dsRNA analogue, is able to induce PKR activation by phosphorylation at Thr 446 (Zhang et al., 2001) and, consistently, its

substrate, eIF2α, is phosphorylated at Ser 51 (Hinnebusch, 2000). This suggests that the effects of HSV1 are not specific to this virus and that other viruses may cause elevated BACE1 levels. However, it should be stressed that HSV1 is uniquely able to cause such changes as it is present in a high proportion of elderly brains. HSV1 is present and is active in a high proportion of brains of elderly normal subjects as well as AD patients (Jamieson et al., 1991; Wozniak et al., 2005). However, PKR was activated only in the brain sample from the AD patient. One possible explanation for this is that there might e genetic variability in the PKR activation that could be related to AD development. Recently a genetic association between rs2254985 polymorphism within *EIF2AK2* gene and the risk of AD has been found (Bullido et al., 2008). In our study we found a trend to association of the same marker with AD susceptibility in a recessive model. However our results show that: i) the at-risk allele (T allele) described herein is the opposite (C allele) to that reported by Bullido and coworkers, ii) according to the original study, the effect of the rs2254985 occurs even in the absence of APOE-ε4 whereas we did not detect any interaction among these variants, iii) contrary to the results reported by Bullido and coworkers, we did not observe any influence of the rs2254985 variant over the age of AD onset. Differences in sample size, mean age of AD onset in patients and characteristics of the control group could account for these discrepancies between the two studies. Also, the opposite effects between our results and Bullido's work could be due to the consequence of linkage disequilibrium between the rs2254985 polymorphism and another "true" risk variant within the gene.

In conclusion, this study presents further evidence for considering that HSV1 is an aetiological factor contributing to sporadic AD. We confirmed that this neurotropic virus can activate PKR, an eIF2α kinase. Activated PKR results in BACE1 translation and increased Aß production. Interestingly, despite the fact that a high proportion of elderly people

harbour HSV1 DNA in brain, PKR is activated only in those of AD patients, making this protein a putative therapeutic target in AD. Other features of the life-cycle of the virus and other factors such as APOE genotype might be involved also in the differential pattern of PKR activation observed within HSV1 infected brains.

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6. Disclosure statement

None of the authors of the present manuscript has any actual or potential conflicts of interest to declare.

7. Ethical statement

All the procedures of the present work using human samples have been previously approved by the Ethical Commite of the Institut Municipal de Investigació Mèdica and University Pompeu Fabra (IMIM-UPF).

FIGURE 1

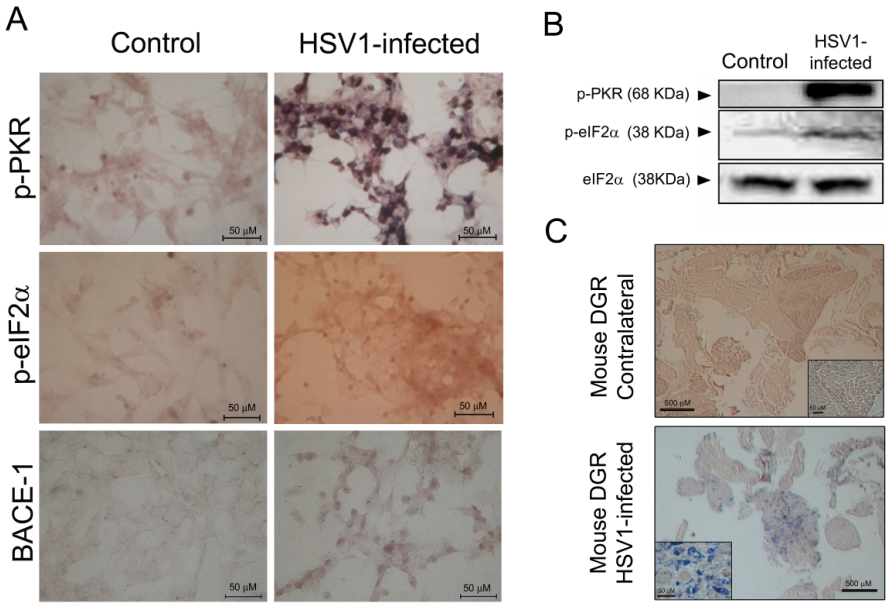

Figure 1. HSV1 infection activates PKR. (A) SH-SY5Y cells were infected with HSV1 (1pfu/cell, 24h) or were not infected (controls). Immunocytochemistry analysis was carried with the following antibodies: anti-p-PKR, anti-p-eIF2α and anti-BACE1. (B) Protein extracts of SH-SY5Y cells infected with HSV1 (3pfu/cell, 24h) and uninfected cells (controls) were analysed by Western blotting using the following antibodies: antip-PKR, anti-p-eIF2α and anti-eIF2α. (C) Sections from mouse dorsal ganglion root (DRG) were obtained from HSV1-infected mice. Contralateral uninfected ganglia were used as controls. Immunohistochemistry analysis was carried out to detect p-PKR.

FIGURE 2

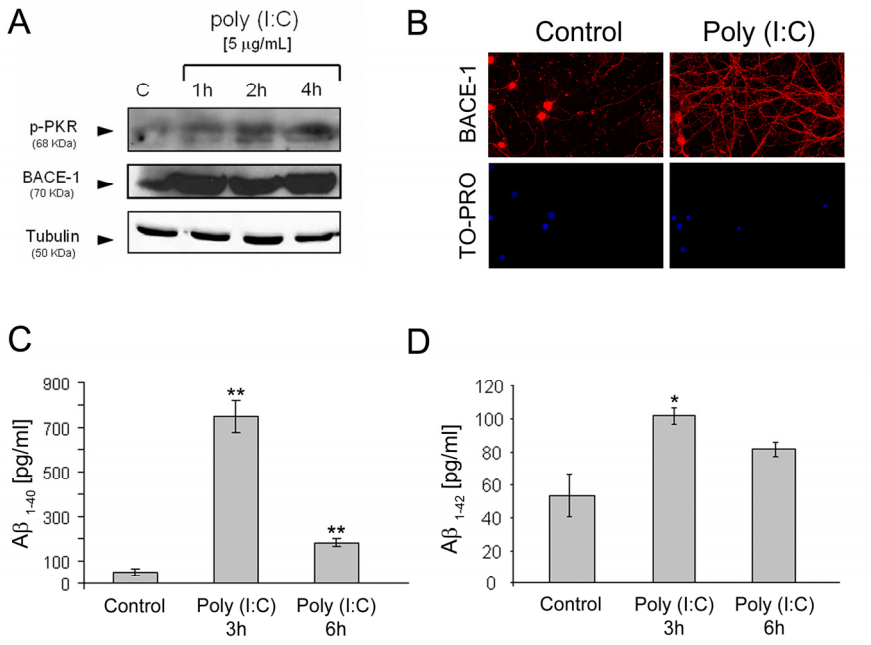

Figure 2. Poly (I:C) induces PKR activation, upregulates BACE1 and promotes amyloidogenesis. (A) The viral dsRNA analog polyinosinic:polycytidylic acid [poly (I:C)] activates PKR in HeLa cells. Protein extracts obtained at increasing times of poly (I:C) treatment were analysed by Western blotting using anti-phospho-PKR(T446), anti-BACE1 and anti-tubulin antibodies. (B) Mature hippocampal neurons were challenged with poly (I:C) for 2 h and analysed by immunofluorescence to detect BACE1 expression levels. Poly (I:C) triggers Aβ1-40 and Aβ1-42 production in HEKAPPsw cells (C, D). HEK-APPsw cells were challenged with poly (I:C) at a concentration of 5 μg/mL. Then after 3h and 6h the media were collected and Aβ1-40 (C) and Aβ1-42 (D) levels were determined. Data are mean ± SEM of 3 independent experiments performed in triplicate. *$p<0.05$ and **$p<0.005$ by Student's *t* test.

FIGURE 3

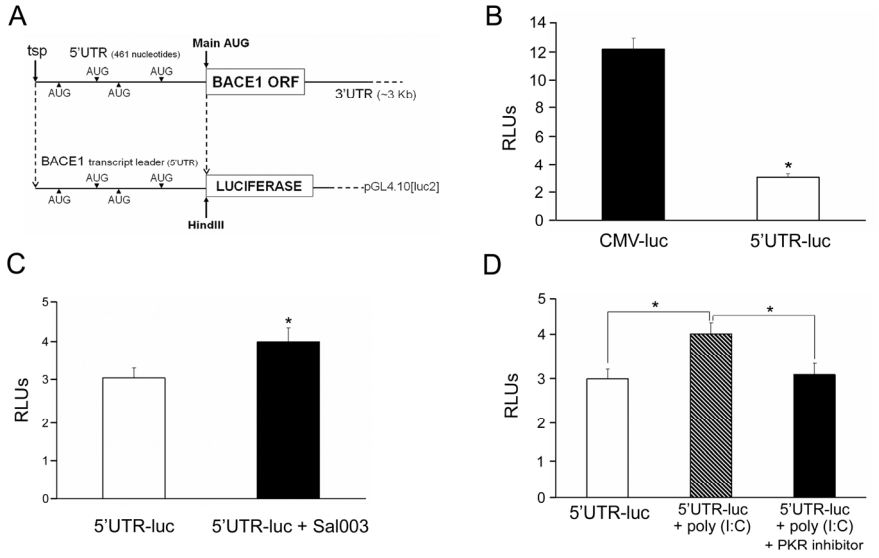

Figure 3. Poly (I:C) induces BACE1 translation in a mechanism depending on PKR catalysed eIF2α phosphorylation. (A) BACE1 5'UTR fragment was inserted into the HindIII site of a luciferase reporter construct (5'UTR-luc). The plasmids bear the strong cytomegalovirus promoter (CMV), which allows sufficient reporter gene expression for luciferase determinations. HeLa cells were transfected with 5'UTR-luc or empty plasmids (named as CMV) for luciferase reporter studies (B, C, D). 5'UTR-luc repressed translation in comparison with the empty 5'UTR-free luciferase construct (B). Inhibition of eIF2α phosphatase PP1c by Sal003 de-repressed the reporter signal elicited by 5'UTR-luc (C). Stimulation (3h) with poly (I:C) also de-repressed the reporter signal yielded by 5'UTR-luc (D). This effect was abolished when cells were preincubated with a specific PKR inhibitor that acts as a potent ATP-binding site directed inhibitor of PKR. Data are mean ± SEM of 3 independent experiments performed in triplicate. *$p<0.05$ by Student's *t* test.

FIGURE 4

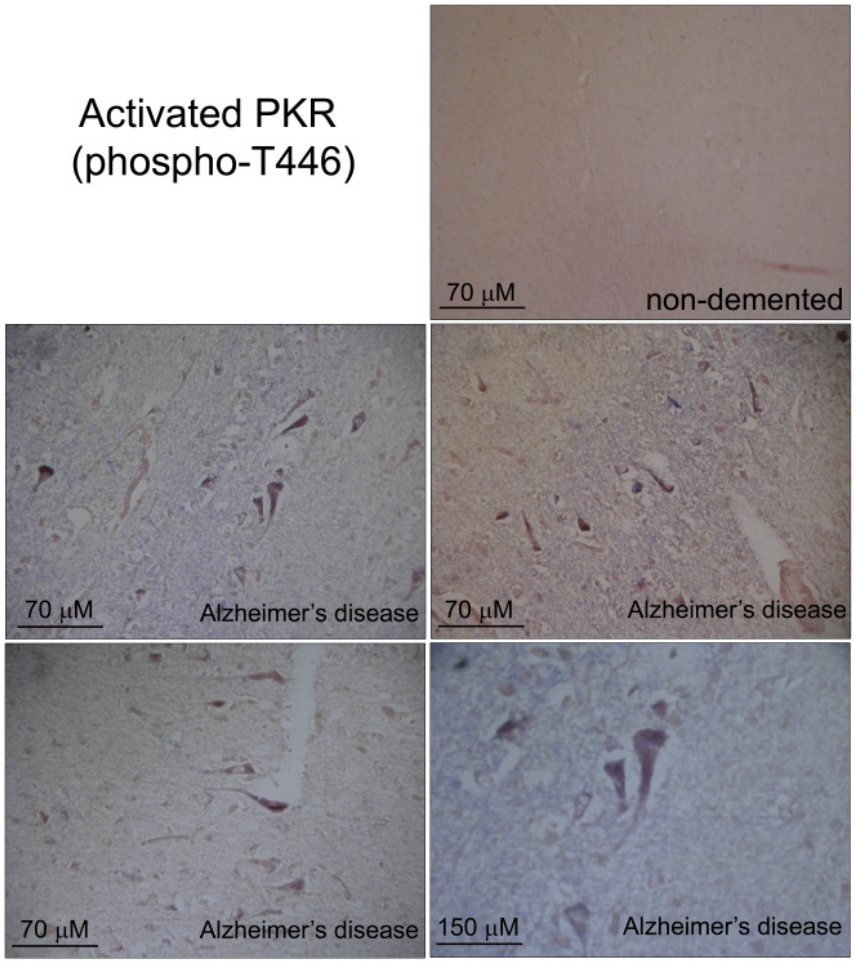

Figure 4. Presence of activated PKR in human brain tissue. The presence of activated PKR -autophosphorylated at Thr 446- was studied in human brain sections (temporal lobe) from one non-demented control and one AD patient by immunohistochemistry. All brain sections analysed were positive for HSV1 infection.

FIGURE 5

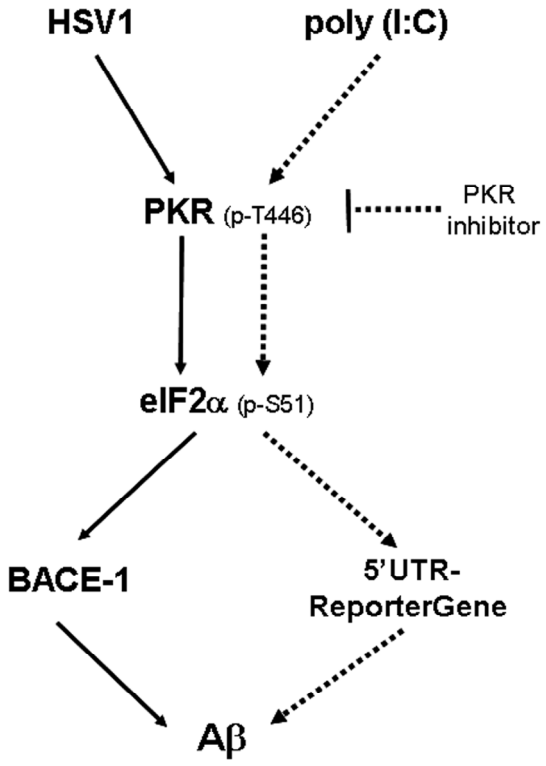

Figure 5. Biochemical pathway linking HSV1 infection and AD. Despite the viral ability to circumvent host defensive mechanisms, including PKR activation, HSV1 activates PKR *in vitro* and *in vivo*. Activated PKR increases eIF2α phosphorylation levels, leading to BACE1 translation de-repression, BACE1 protein up-regulation and Aβ production (left track; continuous line). In the right track (dotted line) we present the pharmacological and biological tools that we used to study this pathway: poly (I:C), to mimic the effect of viral dsRNA; a specific imidazolooxindole compound that acts as a potent PKR inhibitor; and a 5'UTR-luc reporter construct used for the evaluation of the translational effect exerted by the PKR-eIF2α pathway over BACE1 5'UTR.

TABLES

Table 1 Primers and probes employed for the genotyping protocol

Primers
Forward 5'-CCACAGGCACGACAAGCATA-3'
Reverse 5'-CGAGTGATACCAGCGAAGACTAAG-3'
Detection probe
Sensor 5'- GGCATCGAGGTCCATCCC[Flc] -3'
Anchor 5'- [Cy5]TCAGGAGACCCTGGCTATCATAG[Phos] -3'

[Flc], Fluorescein; [Cy5], Cy5 Fluorochrome; [Phos] Phosphate

Table 2. rs2254958 allele and genotype frequencies distribution

	Minor allele frequency	CC	CT	TT	*P* value (HWE)
Controls	0.46	**335 (0.30)**	**550 (0.49)**	**237 (0.21)**	**0.31**
AD cases	0.45	**356 (0.30)**	**606 (0.51)**	**229 (0.19)**	**0.69**

Genotype frequencies are indicated in parenthesis. HWE: Hardy-Weinberg Equilibrium test with one degree of freedom.

Table 3. rs2254958 genotype distribution attending to gender and APOE

	OR	95% CI	*P* value
gender	1.93	1.64-2.34	< 0.001
APOE	3.21	2.65-3.88	< 0.001
Rs2254958 (CC+CT vs TT)	1.24	1.00-1.53	0.05

8. References

Alexopoulou,L., Holt,A.C., Medzhitov,R., and Flavell,R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732-738.

Ball,M.J. (1982). "Limbic predilection in Alzheimer dementia: is reactivated herpesvirus involved?". Can. J. Neurol. Sci. *9*, 303-306.

Bullido,M.J., Martinez-Garcia,A., Tenorio,R., Sastre,I., Munoz,D.G., Frank,A., and Valdivieso,F. (2008). Double stranded RNA activated EIF2 alpha kinase (EIF2AK2; PKR) is associated with Alzheimer's disease. Neurobiol. Aging *29*, 1160-1166.

Cassady,K.A. and Gross,M. (2002). The herpes simplex virus type 1 U(S)11 protein interacts with protein kinase R in infected cells and requires a 30-amino-acid sequence adjacent to a kinase substrate domain. J. Virol. *76*, 2029-2035.

Chang,R.C., Suen,K.C., Ma,C.H., Elyaman,W., Ng,H.K., and Hugon,J. (2002a). Involvement of double-stranded RNA-dependent protein kinase and phosphorylation of eukaryotic initiation factor-2alpha in neuronal degeneration. J. Neurochem. *83*, 1215-1225.

Chang,R.C., Wong,A.K., Ng,H.K., and Hugon,J. (2002b). Phosphorylation of eukaryotic initiation factor-2alpha (eIF2alpha) is associated with neuronal degeneration in Alzheimer's disease. Neuroreport *13*, 2429-2432.

Chou,J., Chen,J.J., Gross,M., and Roizman,B. (1995). Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma 134.5- mutants of herpes simplex virus 1. Proc. Natl. Acad. Sci. U. S. A *92*, 10516-10520.

Citron,M., Oltersdorf,T., Haass,C., McConlogue,L., Hung,A.Y., Seubert,P., Vigo-Pelfrey,C., Lieberburg,I., and Selkoe,D.J. (1992). Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature *360*, 672-674.

Coma,M., Guix,F.X., Ill-Raga,G., Uribesalgo,I., Alameda,F., Valverde,M.A., and Munoz,F.J. (2008). Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells. Neurobiol. Aging *29*, 969-980.

D.Del Toro, M.Coma, I.Uribesalgo, F.X.Guix, and F.J.Muñoz. The amyloid beta-protein precursor and Alzheimer's disease. Therapeutic Approaches. Curr.Med.Chem.-Central Nervous System Agents 5, 271-283. 2009. Bentham Science Publishers Ltd. 2005.

De Pietri,T.D., Mihailovich,M., Di,C.A., Codazzi,F., Grohovaz,F., and Zacchetti,D. (2004). Translational regulation of BACE-1 expression in neuronal and non-neuronal cells. Nucleic Acids Res. *32*, 1808-1817.

de Haro, C., Mendez,R., and Santoyo,J. (1996). The eIF-2alpha kinases and the control of protein synthesis. FASEB J. *10*, 1378-1387.

Der,S.D., Yang,Y.L., Weissmann,C., and Williams,B.R. (1997). A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. Proc. Natl. Acad. Sci. U. S. A *94*, 3279-3283.

Dobson,C.B., Sales,S.D., Hoggard,P., Wozniak,M.A., and Crutcher,K.A. (2006). The receptor-binding region of human apolipoprotein E has direct anti-infective activity. J. Infect. Dis. *193*, 442-450.

Gatta,L.B., Albertini,A., Ravid,R., and Finazzi,D. (2002). Levels of beta-secretase BACE and alpha-secretase ADAM10 mRNAs in Alzheimer hippocampus. Neuroreport *13*, 2031-2033.

Glenner,G.G. and Wong,C.W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. *120*, 885-890.

Hinnebusch, A. G. Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. 184-244. 2000. NY, Cold Spring Harbor Laboratory Press.

Hinnebusch, A. G. Translational control of GCN4: Gene-specific regulation by phosphorylation of eIF2. [Translational Control], 199-244. 1996. Coldspring Harbor Pres, J.W.B. Hershey, M.B. Mathews and N. Sonenberg, Editors.

Hinnebusch,A.G. (1997). Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. J. Biol. Chem. *272*, 21661-21664.

Hussain,I., Powell,D., Howlett,D.R., Tew,D.G., Meek,T.D., Chapman,C., Gloger,I.S., Murphy,K.E., Southan,C.D., Ryan,D.M., Smith,T.S., Simmons,D.L., Walsh,F.S., Dingwall,C., and Christie,G. (1999). Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol. Cell Neurosci. *14*, 419-427.

Itzhaki, R.F., Lin,W.R., Shang, D., Wilcock,G.K., Faragher,B. and Jamieson,G.A. (1997) Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. Lancet.
349, 241-4.

Jamieson,G.A., Maitland,N.J., Wilcock,G.K., Craske,J., and Itzhaki,R.F. (1991). Latent herpes simplex virus type 1 in normal and Alzheimer's disease brains. J. Med. Virol. *33*, 224-227.

Jammi,N.V., Whitby,L.R., and Beal,P.A. (2003). Small molecule inhibitors of the RNA-dependent protein kinase. Biochem. Biophys. Res. Commun. *308*, 50-57.

Kaech,S. and Banker,G. (2006). Culturing hippocampal neurons. Nat. Protoc. *1*, 2406-2415.

Lammich,S., Schobel,S., Zimmer,A.K., Lichtenthaler,S.F., and Haass,C. (2004). Expression of the Alzheimer protease BACE1 is suppressed via its 5'-untranslated region. EMBO Rep. *5*, 620-625.

Lee,S.B. and Esteban,M. (1994). The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology *199*, 491-496.

Lin,X., Koelsch,G., Wu,S., Downs,D., Dashti,A., and Tang,J. (2000). Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. Proc. Natl. Acad. Sci. U. S. A *97*, 1456-1460.

Marcinkiewicz,M. and Seidah,N.G. (2000). Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. J. Neurochem. *75*, 2133-2143.

Matsumoto,M., Funami,K., Oshiumi,H., and Seya,T. (2004). Toll-like receptor 3: a link between toll-like receptor, interferon and viruses. Microbiol. Immunol. *48*, 147-154.

McKhann,G., Drachman,D., Folstein,M., Katzman,R., Price,D., and Stadlan,E.M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology *34*, 939-944.

Mihailovich,M., Thermann,R., Grohovaz,F., Hentze,M.W., and Zacchetti,D. (2007). Complex translational regulation of BACE1 involves upstream AUGs and stimulatory elements within the 5' untranslated region. Nucleic Acids Res. *35*, 2975-2985.

Morris,D.R. and Geballe,A.P. (2000). Upstream open reading frames as regulators of mRNA translation. Mol. Cell Biol. *20*, 8635-8642.

O'Connor,T., Sadleir,K.R., Maus,E., Velliquette,R.A., Zhao,J., Cole,S.L., Eimer,W.A., Hitt,B., Bembinster,L.A., Lammich,S., Lichtenthaler,S.F., Hebert,S.S., De,S.B., Haass,C., Bennett,D.A., and Vassar,R. (2008). Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. Neuron *60*, 988-1009.

Peel,A.L. (2004). PKR activation in neurodegenerative disease. J. Neuropathol. Exp. Neurol. *63*, 97-105.

Peel,A.L. and Bredesen,D.E. (2003). Activation of the cell stress kinase PKR in Alzheimer's disease and human amyloid precursor protein transgenic mice. Neurobiol. Dis. *14*, 52-62.

Preece,P., Virley,D.J., Costandi,M., Coombes,R., Moss,S.J., Mudge,A.W., Jazin,E., and Cairns,N.J. (2003). Beta-secretase (BACE) and GSK-3 mRNA levels in Alzheimer's disease. Brain Res. Mol. Brain Res. *116*, 155-158.

Ramirez,L.R., Boada,M., Saez,M.E., Hernandez,I., Mauleon,A., Rosende,R.M., Martinez,L.P., Gutierrez,M., Real,L.M., Lopez,A.J., Gayan,J., Antunez,C., Gonzalez,P.A., Tarraga,L., and Ruiz,A. (2009). GAB2 Gene Does Not Modify the Risk of Alzheimer's Disease in Spanish APOE e4 Carriers. J. Nutr. Health Aging *13*, 214-219.

Scheuner,D., Patel,R., Wang,F., Lee,K., Kumar,K., Wu,J., Nilsson,A., Karin,M., and Kaufman,R.J. (2006). Double-stranded RNA-dependent protein kinase phosphorylation of the alpha-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. J. Biol. Chem. *281*, 21458-21468.

Sinha,S., Anderson,J.P., Barbour,R., Basi,G.S., Caccavello,R., Davis,D., Doan,M., Dovey,H.F., Frigon,N., Hong,J., Jacobson-Croak,K., Jewett,N., Keim,P., Knops,J., Lieberburg,I., Power,M., Tan,H., Tatsuno,G., Tung,J., Schenk,D., Seubert,P., Suomensaari,S.M., Wang,S., Walker,D., Zhao,J., McConlogue,L., and John,V. (1999). Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature *402*, 537-540.

Sun,X., He,G., Qing,H., Zhou,W., Dobie,F., Cai,F., Staufenbiel,M., Huang,L.E., and Song,W. (2006). Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. Proc. Natl. Acad. Sci. U. S. A *103*, 18727-18732.

Tamagno,E., Bardini,P., Obbili,A., Vitali,A., Borghi,R., Zaccheo,D., Pronzato,M.A., Danni,O., Smith,M.A., Perry,G., and Tabaton,M. (2002). Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol. Dis. *10*, 279-288.

Tan,S.L. and Katze,M.G. (1999). The emerging role of the interferon-induced PKR protein kinase as an apoptotic effector: a new face of death? J. Interferon Cytokine Res. *19*, 543-554.

Taylor,S.S., Haste,N.M., and Ghosh,G. (2005). PKR and eIF2alpha: integration of kinase dimerization, activation, and substrate docking. Cell *122*, 823-825.

Vassar,R., Bennett,B.D., Babu-Khan,S., Kahn,S., Mendiaz,E.A., Denis,P., Teplow,D.B., Ross,S., Amarante,P., Loeloff,R., Luo,Y., Fisher,S., Fuller,J.,

Edenson,S., Lile,J., Jarosinski,M.A., Biere,A.L., Curran,E., Burgess,T., Louis,J.C., Collins,F., Treanor,J., Rogers,G., and Citron,M. (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science *286*, 735-741.

Wek,R.C., Jiang,H.Y., and Anthony,T.G. (2006). Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. *34*, 7-11.

Williams,B.R. (1999). PKR; a sentinel kinase for cellular stress. Oncogene *18*, 6112-6120.

Wozniak,M.A., Frost,A.L., and Itzhaki,R.F. (2009a). Alzheimer's disease-specific tau phosphorylation is induced by herpes simplex virus type 1. J. Alzheimers. Dis. *16*, 341-350.

Wozniak,M.A., Itzhaki,R.F., Shipley,S.J., and Dobson,C.B. (2007). Herpes simplex virus infection causes cellular beta-amyloid accumulation and secretase upregulation. Neurosci. Lett. *429*, 95-100.

Wozniak,M.A., Mee,A.P., and Itzhaki,R.F. (2009b). Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. J. Pathol. *217*, 131-138.

Wozniak,M.A., Shipley,S.J., Combrinck,M., Wilcock,G.K., and Itzhaki,R.F. (2005). Productive herpes simplex virus in brain of elderly normal subjects and Alzheimer's disease patients. J. Med. Virol. *75*, 300-306.

Yan,R., Bienkowski,M.J., Shuck,M.E., Miao,H., Tory,M.C., Pauley,A.M., Brashier,J.R., Stratman,N.C., Mathews,W.R., Buhl,A.E., Carter,D.B., Tomasselli,A.G., Parodi,L.A., Heinrikson,R.L., and Gurney,M.E. (1999). Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature *402*, 533-537.

Zhang,F., Romano,P.R., Nagamura-Inoue,T., Tian,B., Dever,T.E., Mathews,M.B., Ozato,K., and Hinnebusch,A.G. (2001). Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. J. Biol. Chem. *276*, 24946-24958.

Zhang,X., Zhou,K., Wang,R., Cui,J., Lipton,S.A., Liao,F.F., Xu,H., and Zhang,Y.W. (2007). Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. J. Biol. Chem. *282*, 10873-10880.

Zhou,W. and Song,W. (2006). Leaky scanning and reinitiation regulate BACE1 gene expression. Mol. Cell Biol. *26*, 3353-3364.

CHAPTER III

"Nitro-oxidative stress is a key contributor to the neuronal damage induced by amyloid ß-peptide in Alzheimer's disease"

Gerard ILL-Raga, Eva Ramos-Fernández, Francesc X. Guix, Marta Tajes, Mònica Bosch, Ernest Palomer, Juan Godoy, Sebastián Belmar, Waldo Cerpa, James W. Simpkins, Nibaldo C. Inestrosa, and Francisco J. Muñoz

Nitro-oxidative stress is a key contributor to the neuronal damage induced by amyloid ß-peptide in Alzheimer's Disease

Gerard ILL-Raga[1], Eva Ramos-Fernández[1], Francesc X. Guix[1], Marta Tajes[1], Mònica Bosch[1], Ernest Palomer[1], Juan Godoy[2], Sebastián Belmar[2], Waldo Cerpa[2], James W. Simpkins[3], Nibaldo C. Inestrosa[2], and Francisco J. Muñoz[1*]

[1]Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Spain; [2]Centro de Regulación Celular y Patología "Joaquín V, Luco" (CRCP), MIFAB, Centro de Envejecimiento y Regeneración (CARE), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; [3]Department of Pharmacology and Neuroscience, Institute for Aging and Alzheimer's Disease Research, University of North Texas Health Science Center, Fort Worth, Texas, USA.

Running title: Aß-peptide induces nitro-oxidative stress

[*]To whom correspondence should be addressed:
Dr. Francisco J. Muñoz
Grup de Fisiologia Molecular i Canalopaties
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra
C/ Dr. Aiguader, 88, Barcelona 08003, Spain
Fax: +34 93 316 09 01; Phone: +34 93 316 08 52
e-mail: paco.munoz@upf.edu

Abstract

Different mechanisms have been proposed to mediate amyloid ß-peptide (Aß) neurotoxicity in Alzheimer's disease (AD). In the present work we demonstrate that nitro-oxidative stress is the major cytotoxic mechanism induced by Aß. Firstly, we assayed Aß cytotoxicity on yeasts, since these eukaryotic cells are a well known model for H_2O_2 toxicity and they have a cell wall that avoids any direct interaction of Aß fibrils with cell membrane receptors or calcium channels. As expected, we obtained a significant reduction on yeast viability when challenged with Aß fibrils. Furthermore, we assayed the antioxidant trolox as neuroprotective on mouse hippocampal neurons challenged with Aß fibrils. Trolox protected neurons in cell viability studies, maintaining the vesicular transport integrity and avoiding the trigger of apoptotic mechanisms. Interestingly, we have also found that brain derived neuronal factor (BDNF) and neurotrophin-3 (NT-3) were able to protect mouse hippocampal and cortical neurons against H_2O_2 and Aß fibrils. Considering that superoxide anion, produced by Aß cell damage, and nitric oxide, whose production is altered in AD, react to form the highly reactive peroxynitrite anion, we studied the role of Trolox to ameliorate the peroxynitrite cell damage. Finally, one of the major proteins to be nitrotyrosinated in AD, the triose phosphate isomerase (TPI) was assayed searching for a denitrase activity that could revert intracellular nitrotyrosination. We have found that human neuroblastoma SH-SY5Y cells express a constitutive denitrase activity that partially denitrated nitro-TPI. Summarizing, our results support a key role of nitro-oxidative stress in the neuronal damage induced by Aß fibrils.

Key words: Alzheimer's disease; amyloid ß-peptide; oxidative stress; antioxidants; peroxynitrite; triose phosphate isomerase.

Introduction

Alzheimer's disease (AD) is a neurodegenerative process characterized by senile plaques, neurofibrillary tangles and neuronal death. Senile plaques are composed by the amyloid-ß-peptide (Aß), a 40-42 amino acid peptide produced by the proteolytic cleavage of the amyloid precursor protein (APP) [1]. Soluble Aß turns neurotoxic when it misfolds in ß-sheet fibrils [2]. There is a piece of evidence relating the pathophysiology of AD with oxidative stress [3,4]. In fact, Aß fibrils produce hydrogen peroxide [5-7] that initiates an intracellular cascade of reactive oxygen species. Consequently, several studies have reported neuroprotection against Aß mediated cytotoxicity by antioxidants [8].

On the other hand, the superoxide anion produced by the intracellular H_2O_2 action can react with nitric oxide (NO) to yield peroxynitrite. Peroxynitrite is a highly reactive molecule that nitrates the tyrosine residues of proteins impairing its physiological functions [9]. In fact specific cerebral regions of AD patients have higher protein nitrotyrosination levels than controls, especially in the hippocampus and the cerebral cortex [10,11], as well as in cerebroespinal fluid (CSF) proteins [12]. Moreover there is evidence for the involvement of altered NO production in AD since the neuronal NO synthase (nNOS) has an increased expression in those neurons with neurofibrillary tangles in the entorhinal cortex and hippocampus of AD patients [13]. The major nitrotyrosinated proteins in AD are related to glucose metabolism (γ-enolase/α-enolase, lactate deshydrogenase and triosephosphate isomerase; TPI) or cellular cytoskeleton (α-actin) [14-16].

In the present work we have addressed the study of the effect of Aß fibrils in different cell models demonstrating that nitro-oxidative stress is related to the etiopathogeny of AD, and we have searched for protection against Aß-induced damage.

Materials and Methods

Cell lines

The yeasts *Saccaromyces cerevisae* were grown in Petri dishes with YPD-Agar. Rat pheochromocytoma (PC12) cells were grown on collagen-coated dishes in Dulbecco's modified Eagle medium (DMEM; Gibco BRL), 10% fetal bovine serum (FBS), 5% horse serum and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Human neuroblastoma (SH-SY5Y) cells were grown in DMEM supplemented with 15% FBS and antibiotics.

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Primary mouse embryo hippocampal and cortical cell cultures

Hippocampal and cortical cells were isolated from 18-day-old CD1 mouse embryos. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Cortex and hippocampi were aseptically dissected and tripsinized. Cells were seeded in phenol-red-free DMEM plus 10% horse serum into 1% poly-L-Lysine coated plates. After 120 min, medium was removed and neurobasal medium was added containing 1% B27 supplement (Gibco BRL), plus antibiotics. On day 3 of culture, cells were treated with 2 µM 1-ß-D-arabinofuranosylcytosine for 24 h to eliminate proliferating non-neuronal cells. Cultured hippocampal and cortical cells were used for the experiments on day 7.

Aggregation of Aß peptide

Synthetic $Aß_{1-40}$ and $Aß_{1-42}$ corresponding to the human Aß wild type sequence and the $Aß_{40-1}$ peptide with the reverse sequence of the human Aß were obtained from Calbiochem. $Aß_{1-40}$ and $Aß_{1-42}$ fibrils, and $Aß_{40-1}$ aggregates were obtained by dissolving freeze-dried aliquots in dimethyl sulfoxide (DMSO). Peptide stock aliquots were diluted in 0.1 M Tris-HCl (pH 7.4) to a final concentration of 100 µM Aß. Solutions were stirred continuously (1,300 rpm) at room temperature for 48 h. Then the fibrils were maintained at room temperature for 1 week and kept at -20º C before using.

Cell viability assays in S. cerevisae.

S. cerevisae were inoculated in 2 mL of standard YPD medium. Cells were grown during ~12 h at 28º C to obtain a saturated culture (OD600 ~2). Cells were diluted at OD600 ~ 1. Then 12 µL of the cellular suspension were seeded in 96-well plates in 100 µL/well. Challenges with H_2O_2 and Aß fibrils were performed for 2 h at 28º C. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. 12 µL of MTT solution (5 mg/mL) were added at the beginning of the experiment. Cells were collected and centrifuged at 3,000 rpm for 5 min at 4ºC. Pellets were washed with 500 µL PBS and centrifugation at 3,000 rpm for 5 min at 4ºC. Pellets were lysed with 100 µL DMSO and vortex for 10 s. The samples were disposed in 96-well plates and the absorbances at 540 and 650 nm were measured in a spectrophotometer. Control cells were assumed as 100%.

Cell viability assays in cell lines and neuronal cells

PC12 cells were seeded in 96-well plates in serum- and phenol red-free medium plus 2 µM insulin at a density of $4x10^3$ cells/100 µL/well. Hippocampal and cortical cells ($2x10^4$ cells/100 µL/ well) were assayed in

serum- and phenol red-free medium supplemented with B27 without antioxidants. Cells were pre-incubated for 2 h with trolox, brain-derived neurotrophic factor (BDNF; Sigma), neurotrophin-3 (NT-3; Sigma) or neuronal growth factor (NGF; Sigma). Then, PBS (control), H_2O_2, Aß fibrils or 3-morpholino-sydnonimine (SIN-1; a peroxynitrite donor; Sigma) were added to wells. Cells were incubated for 48 (PC12) or 24 h (hippocampal and cortical neurons) at 37ºC. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Briefly, 11 µL of MTT stock solution (5 mg/mL) were added and after 2 h the reaction was stopped with 120 µL of DMSO. MTT reduction was determined in a spectrophotometer at 540 and 650 nm. Control cells were assumed as 100%.

Caspase-3 activity

The caspase-3 activity was evaluated using Ac-Asp-Glu-Val-Asp (DEVD)-pNA (colorimetric substrate; UPSTATE). 1.5 x 10^6 hippocampal neurons were treated as described above, washed with PBS and lysed in 100 µL ice-cold lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1 % CHAPS, 10 % Glycerol, 10 mM DTT). Neuronal extracts were centrifuged and supernatants collected. 50 µg of protein extracts were incubated in reaction buffer 200 µM DEVD-pNA at 37ºC for 24 h. Samples were analyzed with a plate reader by measuring OD at 405 nm.

Immunofluorescence studies

Hippocampal neurons were plated on polylysine-coated coverslips (2x10^4 cells cells/cover). After 7 days in neurobasal medium, neurons were preincubated with Trolox for 2h and then challenged with $Aß_{1-40}$ fibrils. Neurons were fixed with 4% paraformaldehyde and permeabilized with

0.1% Triton X-100. Immunostaining was performed with rabbit polyclonal anti-SV2 antibody (Ab) (1:300) (Sigma,) Alexa568-bound as secondary Ab (1:5000) (Sigma) and Phalloidin-FITC (Affinity Bio Reagents Inc). Coverslips were mounted and analysed using a Zeiss Confocal microscope.

Apoptosis detection by TUNEL technique

Programmed cell death was evaluated by the TUNEL method using *In Situ* POD Cell Death Detection Kit (Roche). Hippocampal neurons were plated on poly-lysine-coated coverslips ($5x10^4$ cells/cover). After 7 days neurons were preincubated with Trolox for 2 h and then challenged with $Aß_{1-40}$ fibrils for 6 h. Cells were fixed in 4% paraformaldehyde in PBS and permeabilyzed with 0.1 % Triton X-100 in 0.1% sodium citrate. The staining was done as described by the manufacturer. Samples were analysed by light microscope.

Reduced glutathione (GSH) measurement

Hippocampal and cortical cells ($2x10^4$ cells/100 µL/ well in 96-well plates) were assayed in serum- and phenol red-free medium supplemented with B27 without antioxidants. Cells were pre-incubated for 2 h with NGF. Then, PBS (control) or H_2O_2 were added to wells. Cells were incubated for 180 min or 24 h at 37ºC. Then cells were incubated with 40 µM monoclorobimane (MCB; Calbiochem) solution and incubated at RT for 20 min. Cells were washed and the fluorescence was determined in a spectrofluorimeter at 361 excitation and 540 emission. Control cells were assumed as 100%.

Mice brain samples

Mice samples were used following the procedure approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-

Universitat Pompeu Fabra. Cerebral paraffined slides from APPswe/PS1 L166P mice were deparaffined with Clear Rite 3 and subsequent washes with decreasing ethanol dilutions. The Tyramide Signal Amplification kit (PerkinElmer) was used for the immunofluorescence, following the manufacturer instructions. Briefly, slides were incubated 7 min with a 70 % formic acid solution in order to expose the epitopes. After 1h of quenching endogenous peroxidase activity with a 30 % H_2O_2 solution, slides were blocked 1 h at RT with 2% normal serum in TNB blocking buffer. The primary Ab was 1:100 anti-nitrotyrosine (Abcam). The secondary Ab was 1:500 Alexa568-bound rabbit Ab. Slides were maintained by 6 min with the Tyr-FITC reagent at darkness (1:50 in 1x Reagent Diluent). All the washes were performed with TNT 1x buffer.

Denitrase activity study

Non-treated or 100 μM sodium nitroprusside (SNP; a nitric oxide donor; Sigma) treated SH-SY5Y cells were lysed at 4°C with NP40 lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 0.05% aprotinin) containing protease inhibitors (Complete mini-EDTA free) from Roche Diagnostics GmbH. No reducing agent was added in order to maintain the conformational state of the putative denitrase activity. Insoluble fraction was spin down by 15 min centrifugation at 4 ºC and 10.000 rpm. Proteins in the supernatant were quantified with the Bradford assay. Rabbit triose phosphate isomerase (TPI; 1.25 μg/μl) was dissolved in PBS (pH 7.4–7.6) and exposed to 50 mM SIN-1. The nitration process was carried out by shaking at 300 rpm for 3 h at room temperature. Nitro-TPI was mixed with loading buffer, run in a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (2h at R.T.) for western blot analysis. Denitrase activity was assayed by incubation of nitrocellulose membranes containing rabbit nitro-TPI with 1 ml SH-SY5Y cell lysates or PBS under

shacking at 37ºC for 1 h before revealing. Extra protease inhibitors were added to ensure that decline of the nitrotyrosination signal was not due to degradation of the protein. The glutathione S-transferase (GST) inhibitor ethacrynic acid (10 μM) was probed in some SH-SY5Y lysates since this enzyme has been claimed to have denitrase activity. The membranes were assayed with a rabbit anti-nitrotyrosine polyclonal Ab (1:1000, Invitrogen) and an HRP-conjugated anti-rabbit antibody (1:5000, Amersham Bioscience) as a secondary antibody. Bands were visualized using the enhanced chemiluminescence substrate (Super Signal; Pierce) and the Hyperfilm ECL kit from Amersham Bioscience.

TPI immunoprecipitation from human samples

Total protein (500 μg) from brains lysates were incubated with 1.25 μg of anti-TPI polyclonal antibody overnight at 4°C. Following the addition of protein G immobilized on sepharose (Sigma), samples were shaken for 2 h at room temperature. Aggregates were pulled down by centrifugation at 10 000 r.p.m. for 10 min and washed thrice. Protein G and antibody were removed from the immunoprecipitated proteins by boiling the samples for 6 min at 100°C.

Statistical analysis

Data were expressed as the mean ± SEM of the values from the number of experiments as indicated in the corresponding figures. Data were evaluated statistically by using the Student´s *t*-test.

Results

Aβ fibrils are toxic for yeasts

The Aβ fibrils are reported to produce cytotoxicity by the production of H_2O_2 [17] but different receptors and calcium channels have been proposed as the major cytotoxic effect of Aβ fibrils. We performed

experiments of cytotoxicity in yeasts because they are eukaryotic cells but with a cell wall that avoids the interaction of the Aß fibrils with the plasmatic membrane and their putative receptors or channels. *S. cerevisae* are described to be damaged by H_2O_2 [18] and we have found that under our experimental conditions increasing concentrations of H_2O_2 induce cell death in *S. cerevisae* (Fig. 1A). Furthermore, we assayed different concentrations of Aß fibrils. At 175 µM of both $Aß_{1-40}$ or $Aß_{1-42}$ fibrils the yeast cells showed a reduction in the cell viability (P<0.05; Fig. 1B). This high concentration of fibrils correlated with the high concentrations of H_2O_2 (Fig. 1A) needed to cross the yeast cell wall to induce cytotoxicity.

Aß fibrils are toxic due to its amyloid structure

Aß fibrils produce H_2O_2 due to their specific amyloid structure [6]. Consequently, we have obtained that $Aß_{40-1}$, a synthetic peptide with the reverse sequence of the wild type that aggregates but does not form amyloid-like structures, is not toxic for PC12 cells (Fig. 2) compared to $Aß_{1-40}$ fibrils (P<0.005).

Antioxidant protects against $Aß_{1-40}$ fibrils in hippocampal cells

Hippocampal neurons showed a reduction in the cell viability when treated with H_2O_2 (Fig. 3A), which can be prevented by the antioxidant Trolox (P<0.05). Similar protection was obtained with Trolox when cells were challenged with Aß fibrils (Fig. 3B-D). Oxidative stress has been reported to induce apoptosis by the caspase cascade. We studied the activation of the initial caspase of the apoptotic cascade, the caspase 3, by Aß action (Fig. 3B). We obtained that $Aß_{1-40}$ fibrils induced a rapid increase in the activity of caspase 3 correlating with the induction of apoptosis obtained by TUNEL (Fig. 3C). The antioxidant Trolox can prevent the activation of caspase 3 induced by $Aß_{1-40}$ fibrils (Fig. 3B), avoiding the apoptotic process as observed by TUNEL (Fig. 3C).

Neuronal function is associated to its neurite structure and their capability to transport the vesicles with new synthesized components and neurotransmitters from the soma. We have found that $A\beta_{1-40}$ fibrils impairs de vesicle trafficking across the neurite network by the immunolabelling of the synaptic vesicle protein 2 (SV-2) (Fig. 3D). Interestingly, the antioxidant Trolox can maintain the physiological vesicle transport in the neurite network similar to that observed in the controls. These functional patterns correlated with the triggering of apoptosis by $A\beta_{1-40}$ fibrils (Fig. 3B-C).

BDNF and NT-3 protection against Aß

Different molecules able to enhance the intracellular antioxidant protection have been reported as protective in AD (Singh et al., 2006; 2008). We have addressed the study of neurotrophin protection in AD since BDNF is strongly expressed in the hippocampus, as well as in other nervous regions, where it plays an essential neuroprotective and neurotrophic role [19] and NT-3 plays a well-described neurotrophic role in the brain [20]. In the present study, we have observed that BDNF and NT-3 were able to protect hippocampal and cortical neurons against the H_2O_2 insult (Fig. 4A). Both neurotrophin lack of antioxidant properties by themselves but BDNF has been reported to activate the antioxidant intracellular defences [21], that could be acting in the obtained protection. Consequently, we have observed that both BDNF and NT-3 (Fig. 4B) protected hippocampal and cortical neurons against the $A\beta_{1-40}$ fibril challenge ($P<0.05$; $P<0.005$) as we obtained previously with the antioxidant Trolox (Fig. 3B-D).

NGF is synthesized in the cortex and the hippocampus, and it has been reported as both neuroprotective and proapoptotic [22,23]. We performed experiments at different concentrations of NGF (10, 50, 100 and 200

ng/mL; Fig 4C shows data with 50 ng/mL) and no protection was obtained against neither H_2O_2 nor $A\beta_{1-40}$ fibril challenge. This lack of protection could be due to the absence of effect on the intracellular antioxidant system since NGF did not increase the intracellular levels of reduced glutathione (GSH) (Fig. 4D).

Nitrotyrosination in AD

The formation of peroxynitrite by the action of Aß yields to protein nitrotyrosination impairing their normal function. Nitrotyrosination has been widely reported in AD brains [10,11]. Consequently, double transgenic mice overexpressing both APP and PS1 showed nitrotyrosinated proteins in hippocampus (Fig. 5A). One of the major proteins to be nitrotyrosinated in AD is the TPI [15,16], a glycolitic enzyme controlling the glucose metabolism through its isomerase activity. As expected, TPI immunoprecipitated from AD brain was highly nitrotyrosinated in comparision with TPI immunoprecipitated from healthy individual (Fig. 5B).

In order to search for a protection against peroxynitrite, cortical neurons were challenged with the peroxynitrite donor SIN-1 (Fig. 5C). We obtained a protective effect with 1 mM Trolox when low concentrations of SIN-1 (100 μM) were used ($P<0.05$). When we used 500 μM SIN-1 there was not any protective activity of the antioxidant. We carried out experiments with higher concentrations of Trolox but no protective effect was obtained neither.

Since the treatments with NO inhibitors to prevent peroxynitrite formation is not a possible therapeutic approach in AD due to the beneficial role of NO in a plethora of physiological functions, we addressed the study of a previously proposed denitrating activity [24,25] that could play protective roles in nitro-oxidative processes in brain. We

have found that neuroblastoma cells (SH-SY5Y cells) have a non identified enzyme or enzymes with *denitrase* activity (Fig. 5D). To uncover the *denitrase* activity we have incubated nitroTPI as a substrate with cell lysates. We have found that a constitutive denitrase activity existed in neuronal cells since the band of nitroTPI was lower than the control treated with PBS. Interestingly, pretreatment of SH-SY5Y cells with 100 μM SNP (a nitric oxide donor) enhanced this activity, suggesting that cells increased the denitrase activity when a nitrative stress was induced. Since GST has been proposed to have denitrase activity [26], we performed the same experiment but using the GST inhibitor ethacrynic acid (Fig. 5E). The co-incubation of nitroTPI containing membrane with the SNP-pretreated SH-SY5Y lysate and ethacrynic acid abolished the denitrase activity, pointing out to the putative role of GST as a denitrase enzyme in these cells.

Discussion

In the present work we have studied the nitro-oxidative role of Aß in AD. Thus we have demonstrated that Aß fibrils are toxic to cells independently of their interaction with any receptor or calcium channels since yeasts are eukaryotic cells but they are surrounded by a cell wall that avoids the binding to key membrane proteins. *S. cerevisae* is a well-known model to study the effect of oxidative stress triggered by exogenous H_2O_2 [18], which can cross the cell wall as it could be happening with the H_2O_2 generated by Aß fibrils [6].

Furthermore we have found that Trolox, an analog of vitamin E, showed neuroprotective effects against the cytotoxicity induced by both H_2O_2 and Aß fibril. The protective properties of Trolox as free radical scavenger prevent the down-stream effects of the proapoptotic machinery by the fibrilar Aß-induced oxidative stress, and permit to maintain the well-function of the neurite network as well as the neuronal vesicle transport.

In fact a decrease in the number of vesicles transported by neurons in the hippocampus of AD brains has been reported [27].

On the other hand, neurotrophins regulate neuronal development and they are also implicated in the survival, maintenance, repairing, synaptic function and plasticity of the adult nervous system [28]. These molecules are able to bind a dual receptor system consisting of Trk receptor tyrosine kinases and the structurally unrelated p75 neurotrophin receptor (p75NTR) to modulate diverse and sometimes opposing biological actions [23]. In AD brains there is a decrease in neurotrophins and their receptors [29], rendering the neurons more sensitive to Aß-mediated cytotoxicity. Neurotrophins show different distributions through the central (CNS) and the peripheral nervous system (PNS). NT-3 is especially active in hippocampus where could be promoting neurite outgrowth and axonal branching [20] and the survival of cholinergic neurons [30]. Moreover, the highest levels of NGF and BDNF mRNA in the adult mouse brain are found in the hippocampus and both are also widely found in the cerebral cortex [31,32]. Interestingly, BDNF induces an increase in the expression of antioxidant enzymes [21] which could be the explanation for the protection obtained in the present work in hippocampal and cortical neurons when they were challenged with H_2O_2 or Aß fibrils. The lack of NGF protection against Aß-mediated cytotoxicity could be related to the direct effect of Aß on the p75 NGF receptor, since p75 has been related with the triggering of apoptotic processes and the activation of p75 by Aß has been reported [23]. Moreover, it has been described that Aß decreases trk-A (receptor for NGF) expression however increases p75 mRNA [33].

Regarding nitro-oxidative stress, we showed that AD cortex and double transgenic mice overexpressing APP and PS1 have protein nitrotyrosination. One of these proteins is TPI that has been recently reported to be a key protein in the Aß mediated cytotoxicity. The nitrotyrosination of TPI yields to a decrease in the glycolitic flow,

increased methylghlyoxal production and triggers the formation of paired helicoidal filaments of tau protein [16]. Then nitrotyrosination of proteins should be avoided to allow the cell survival. Unfortunately the antioxidant Trolox can only act as a protective agent when the amount of peroxynitrite is low. NO synthesis cannot be inhibited due to its wide physiological roles. This makes the search for a denitrase activity capable of reverting nitrotyrosination an interesting therapeutic approach. Using the nitro-TPI as substrate for the denitrase activity we have found that this activity is present constitutively in neuroblastoma cells and can be increased by NO stimulation. The GST could be the responsible for the denitrase activity, but further work is needed to clarify this point.

Summarizing, antioxidants or the agents that prevent nitro-oxidative stress may play a critical role as neuroprotective agents in the neurodegeneration associated to AD. The nitrotyrosination of intracellular proteins is another target to evaluate therapeutic approaches, especially the TPI nitrotyrosination.

Acknowledgements

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FIGURE 1

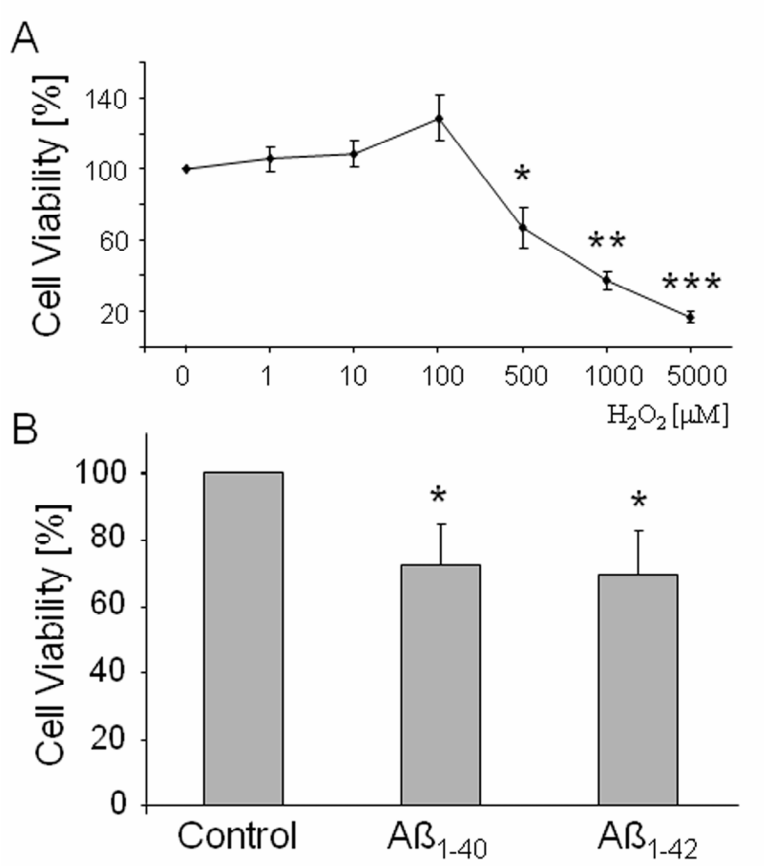

Figure 1. Cytotoxicity of Aß fibrils in yeasts. (**A**) *S. cerevisae* were treated with increasing concentrations of H_2O_2 for 2 hours. Data are mean ± SEM values of 9 independent experiments performed in triplicate. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$ *vs* control (0 µM H_2O_2). (**B**) *S. cerevisae* were treated with 175 µM $Aß_{1-40}$ and $Aß_{1-42}$ fibrils for 2 hours. Data are mean ± SEM values of 3 independent experiments performed in triplicate. * $P<0.05$ *vs* control.

FIGURE 2

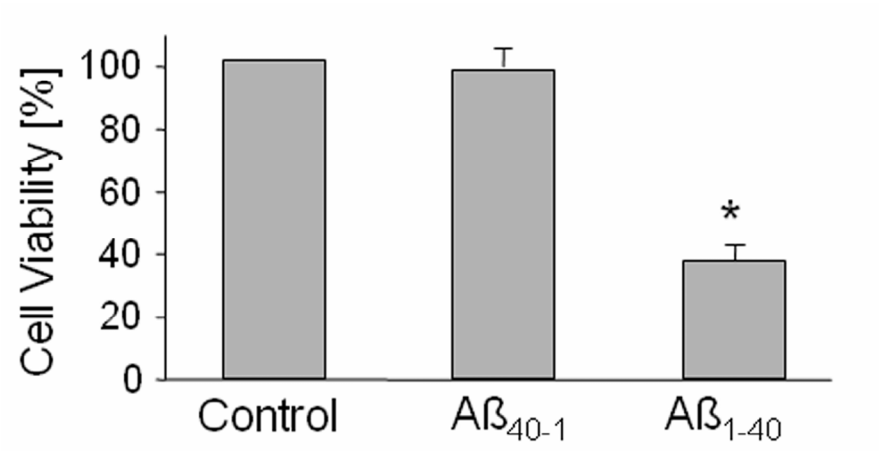

Figure 2. Amyloid structure is needed to induce cytotoxicity. PC12 cells were treated with 10 μM Aß40-1 aggregates and Aß1-40 fibrils for 48 hours. Data are mean ± SEM values of 6 independent experiments performed in triplicate. * $P<0.005$ *vs* control.

FIGURE 3

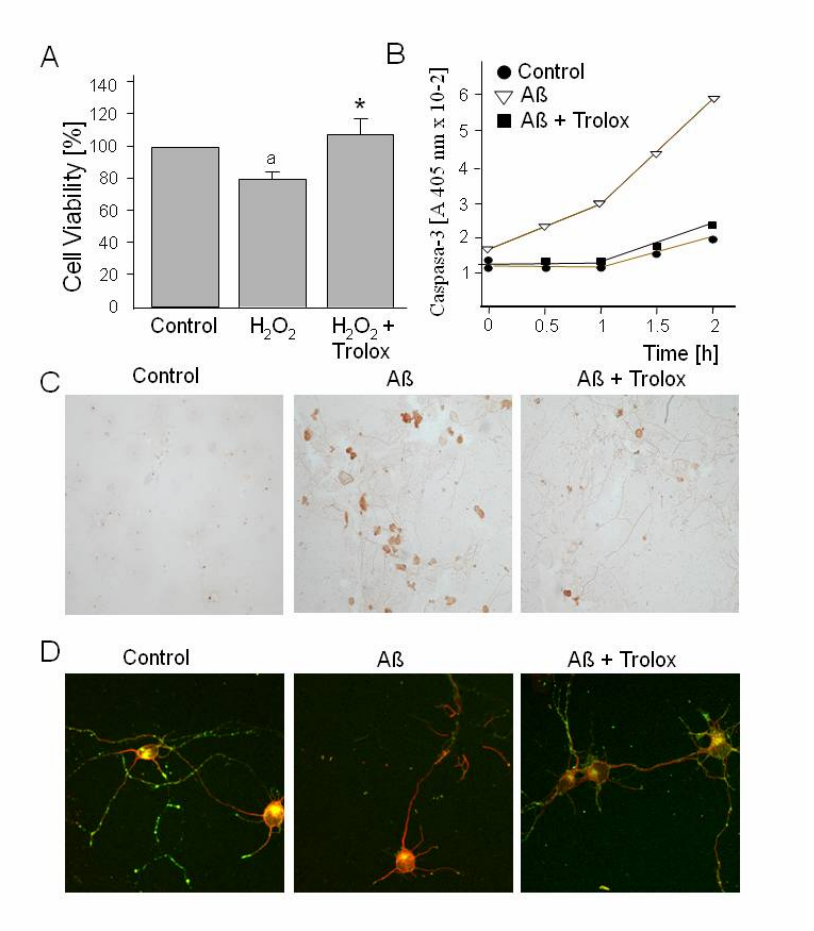

Figure 3. Antioxidant protection against Aß challenge. (**A**) Hippocampal neurons were preincubated with 500 µM Trolox for 2 h and then treated with 100 µM H_2O_2 for 24 h. Data are mean ± SEM values of 6 independent experiments performed in triplicate. * $P<0.05$, [a] $P<0{,}005$. (**B**) Hippocampal neurons were preincubated with 100 µM Trolox for 2 h and treated with 10 µM $Aß_{1-40}$ for 2 h. Caspase-3 activity was quantified with a chromogenic substrate and by measuring the OD at 405 nm. (**C**) Hippocampal neurons were preincubated with 100 µM Trolox for 2 h and treated with 10 µM $Aß_{1-40}$ for 8 h. Apoptotic cells (brown colour) were detected by TUNEL staining method. (**D**) Hippocampal neurons were preincubated with 100 µM Trolox for 2 h and treated with 10 µM $Aß_{1-40}$ for 6 h. The integrity of vesicle trafficking was detected with anti-SV2 antibody (green fluorescence) Neurites were immunodetected with phalloidin-FITC (red fluorescence).

FIGURE 4

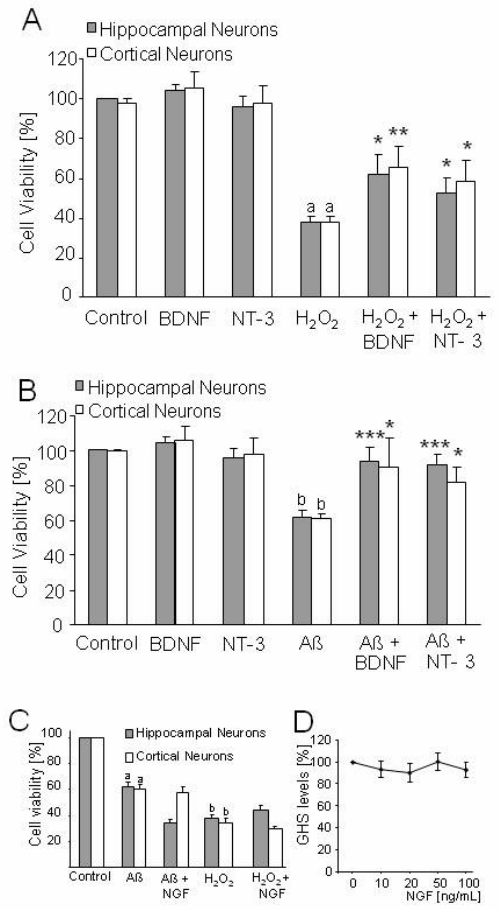

Figure 4. Neurotrophin protection against Aß challenge. (**A, B**) Hippocampal and cortical neurons were preincubated with 50 ng/mL BDNF or NT-3 and challenged with 75 µM H_2O_2 (**A**) or 10 µM $Aß_{1-40}$ (**B**) for 24 h. Cell viability was measured by MTT reduction. Data are mean ± SEM values of 6 to 11 independent experiments performed in triplicate. * P<0.05, ** P<0.005, *** P<0.001, [a] P<0,0001, [b] P<0,001. (**C**) Hippocampal and cortical neurons were preincubated with 50 ng/mL NGF and challenged with 10 µM $Aß_{1-40}$ or 75 µM H_2O_2 for 24 h. Cell viability was measured by MTT reduction. Data are mean ± SEM values of 3 to 11 independent experiments performed in triplicate. [a] P<0,001, [b] P<0,0001. (**D**) Hippocampal and cortical neurons were treated with increasing concentrations of NGF for 24 h. GSH levels were quantified by the measurement of MCB fluorescence. Data are mean ± SEM values of 4 independent experiments performed in triplicate.

FIGURE 5

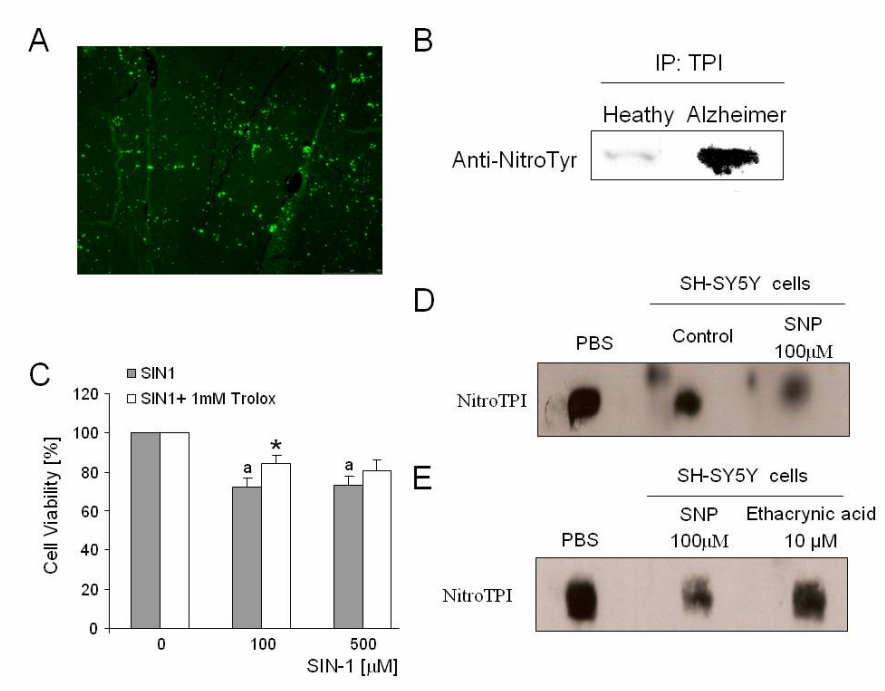

Figure 5. Protein nitrotyrosination in Alzheimer's disease. (**A**) Hippocampus section from double transgenic mice overexpressing APP and PS1 incubated with an anti- nitrotyrosine antibody (green fluorescence). (**B**) Western blot of brain lysates from the cortex of healthy and AD patient immunoprecipitated with an anti-triose phosphate isomerase (TPI) antibody. The blots were revealed with anti-nitrotyrosine antibody. (**C**) Cortical neurons were preincubated with 1 mM Trolox for 2 h and challenged with SIN-1 (a peroxynitrite donor). Cell viability was measured by MTT reduction. Data are mean ± SEM values of 3 independent experiments performed in triplicate. * P<0.05. (**D**) NitroTPI-containing membranes were incubated with SH-SY5Y cell lysates and with PBS alone searching for a denitrase activity. SH-SY5Y cells were previously stimulated with SNP (a nitric oxide donor). (**E**) Same experiment than (**D**), but using the glutathione S-transferase inhibitor ethacrynic acid.

FIGURE 6

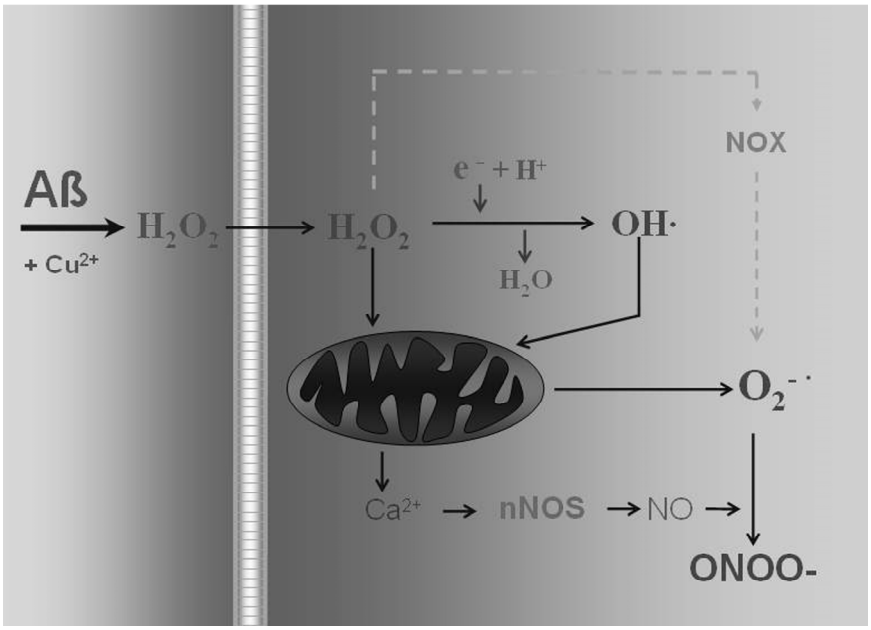

Figure 6. Nitro-oxidative stress in AD. Aß fibrils are producing H_2O_2 by the redox action of Cu^{2+}. H_2O_2 takes an electron to deliver hydroxyl radical acting as a strong local oxidant. It produces damages in mitochondria uncoupling the respiratory chain which yields to the production of superoxide anion. Moreover H_2O_2 could damage the Ca^{2+} channels of the membrane and the intracellular stores, inducing an increase of the intracellular Ca^{2+} that activates nNOS and NO release. NO reacts with superoxide anion to form the highly reactive peroxynitrite anion that induces the nitrotyrosination of the cell proteins. The activation of NADPH oxidase (NOX) could also yield to an enhanced superoxide production.

References

[1] D.Del Toro, M.Coma, I.Uribesalgo, F.X.Guix, F.J.Munoz (2005) The amyloid beta-protein precursor and Alzheimer's disease. Therapeutic Approaches. *Curr Med Chem - Central Nervous System Agents* **5**, 271-281.

[2] Pike CJ, Ramezan-Arab N, Cotman CW (1997) Beta-amyloid neurotoxicity in vitro: evidence of oxidative stress but not protection by antioxidants. *J Neurochem* **69**, 1601-1611.

[3] Lovell MA, Ehmann WD, Butler SM, Markesbery WR (1995) Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* **45**, 1594-1601.

[4] Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N (1996) Oxidative damage in Alzheimer's. *Nature* **382**, 120-121.

[5] Ali FE, Separovic F, Barrow CJ, Cherny RA, Fraser F, Bush AI, Masters CL, Barnham KJ (2005) Methionine regulates copper/hydrogen peroxide oxidation products of Abeta. *J Pept Sci* **11**, 353-360.

[6] Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI (1999) The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* **38**, 7609-7616.

[7] Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, Stokes KC, Leopold M, Multhaup G, Goldstein LE, Scarpa RC, Saunders AJ, Lim J, Moir RD, Glabe C, Bowden EF, Masters CL, Fairlie DP, Tanzi RE, Bush AI (1999) Cu(II) potentiation of alzheimer

abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. *J Biol Chem* **274**, 37111-37116.

[8] Quintanilla RA, Munoz FJ, Metcalfe MJ, Hitschfeld M, Olivares G, Godoy JA, Inestrosa NC (2005) Trolox and 17beta-estradiol protect against amyloid beta-peptide neurotoxicity by a mechanism that involves modulation of the Wnt signaling pathway. *J Biol Chem* **280**, 11615-11625.

[9] Guix FX, Uribesalgo I, Coma M, Munoz FJ (2005) The physiology and pathophysiology of nitric oxide in the brain. *Prog Neurobiol* **76**, 126-152.

[10] Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* **17**, 2653-2657.

[11] Hensley K, Maidt ML, Yu Z, Sang H, Markesbery WR, Floyd RA (1998) Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J Neurosci* **18**, 8126-8132.

[12] Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C (1999) Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. *Neurosci Lett* **269**, 52-54.

[13] Thorns V, Hansen L, Masliah E (1998) nNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. *Exp Neurol* **150**, 14-20.

[14] Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA (2003) Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* **85**, 1394-1401.

[15] Coma M, Guix FX, Uribesalgo I, Espuna G, Sole M, Andreu D, Munoz FJ (2005) Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. *Brain* **128**, 1613-1621.

[16] Guix FX, Ill-Raga G, Bravo R, Nakaya T, de FG, Coma M, Miscione GP, Villa-Freixa J, Suzuki T, Fernandez-Busquets X, Valverde MA, de SB, Munoz FJ (2009) Amyloid-dependent triosephosphate isomerase nitrotyrosination induces glycation and tau fibrillation. *Brain* **132**, 1335-1345.

[17] Behl C, Davis JB, Lesley R, Schubert D (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**, 817-827.

[18] Owsianowski E, Walter D, Fahrenkrog B (2008) Negative regulation of apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1303-1310.

[19] Marmigere F, Givalois L, Rage F, Arancibia S, Tapia-Arancibia L (2003) Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* **13**, 646-655.

[20] Labelle C , Leclerc N (2000) Exogenous BDNF, NT-3 and NT-4 differentially regulate neurite outgrowth in cultured hippocampal neurons. *Brain Res Dev Brain Res* **123**, 1-11.

[21] Onyango IG, Tuttle JB, Bennett JP, Jr. (2005) Brain-derived growth factor and glial cell line-derived growth factor use distinct intracellular signaling pathways to protect PD cybrids from H2O2-induced neuronal death. *Neurobiol Dis* **20**, 141-154.

[22] Salehi A, Delcroix JD, Swaab DF (2004) Alzheimer's disease and NGF signaling. *J Neural Transm* **111**, 323-345.

[23] Hashimoto Y, Kaneko Y, Tsukamoto E, Frankowski H, Kouyama K, Kita Y, Niikura T, Aiso S, Bredesen DE, Matsuoka M, Nishimoto I (2004) Molecular characterization of neurohybrid cell death induced by

Alzheimer's amyloid-beta peptides via p75NTR/PLAIDD. *J Neurochem* **90**, 549-558.

[24] Gorg B, Qvartskhava N, Voss P, Grune T, Haussinger D, Schliess F (2007) Reversible inhibition of mammalian glutamine synthetase by tyrosine nitration. *FEBS Lett* **581**, 84-90.

[25] Leger CL, Torres-Rasgado E, Fouret G, Carbonneau MA (2008) First evidence for an LDL- and HDL-associated nitratase activity that denitrates albumin-bound nitrotyrosine--physiological consequences. *IUBMB Life* **60**, 73-78.

[26] Kuo WN, Kanadia RN, Shanbhag VP, Toro R (1999) Denitration of peroxynitrite-treated proteins by 'protein nitratases' from rat brain and heart. *Mol Cell Biochem* **201**, 11-16.

[27] Coleman PD , Yao PJ (2003) Synaptic slaughter in Alzheimer's disease. *Neurobiol Aging* **24**, 1023-1027.

[28] Kaplan DR , Miller FD (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10**, 381-391.

[29] Michalski B , Fahnestock M (2003) Pro-brain-derived neurotrophic factor is decreased in parietal cortex in Alzheimer's disease. *Brain Res Mol Brain Res* **111**, 148-154.

[30] Durany N, Michel T, Kurt J, Cruz-Sanchez FF, Cervas-Navarro J, Riederer P (2000) Brain-derived neurotrophic factor and neurotrophin-3 levels in Alzheimer's disease brains. *Int J Dev Neurosci* **18**, 807-813.

[31] Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde YA (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J* **9**, 2459-2464.

[32] Dawbarn D, Allen SJ, Semenenko FM (1988) Immunohistochemical localization of beta-nerve growth factor receptors in the forebrain of the rat. *Brain Res* **440**, 185-189.

[33] Olivieri G, Otten U, Meier F, Baysang G, mitriades-Schmutz B, Muller-Spahn F, Savaskan E (2002) Oxidative stress modulates tyrosine kinase receptor A and p75 receptor (low-affinity nerve growth factor receptor) expression in SHSY5Y neuroblastoma cells. *Neurol Clin Neurophysiol* **2002**, 2-10.

CHAPTER IV

"Protein nitrotyrosination in ischaemic stroke"

Gerard ILL-Raga, Francesc X. Guix, Eva Ramos-Fernández, Ernest Palomer, Mònica Bosch, Jordi Jiménez-Conde, Angel Ois, Fernando Pérez-Asensio, Mario Reyes-Navarro, Francesc Alameda, Carlos Opazo, Anna Planas, Jaume Roquer, Miguel A. Valverde, and Francisco J. Muñoz

Protein nitrotyrosination in ischaemic stroke

Gerard ILL-Raga[1] , Francesc X. Guix[1], Eva Ramos-Fernández[1], Ernest Palomer[1], Mònica Bosch[1], Jordi Jiménez-Conde[2] , Angel Ois[2], Fernando Pérez-Asensio[3], Mario Reyes-Navarro[4], Francesc Alameda[5], Carlos Opazo[4], Anna Planas[3], Jaume Roquer[2], Miguel A. Valverde[1], and Francisco J. Muñoz[1*]

[1]Laboratory of Molecular Physiology and Channelopathies, Universitat Pompeu Fabra, Barcelona, Catalonia, Spain; [2]Servei de Neurologia, Hospital del Mar-IMIM, Barcelona; [3]Institut d'Investigacions Biomèdiques de Barcelona (IIBB)-Consejo Superior de Investigaciones Científicas (CSIC), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); [4]Laboratory of Neurobiometals, Department of Physiology, University of Concepción, Chile; [5]Servei d'Anatomia Patologica, Hospital del Mar-IMIM, Barcelona.

Running title: *Nitrotyrosination in stroke*

[*]To whom correspondence should be addressed:
Dr. Francisco J. Muñoz
Grup de Fisiologia Molecular i Canalopaties
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra
Parc de Recerca Biomèdica de Barcelona,
C/ Dr. Aiguader, 88, Barcelona 08003, Catalonia, Spain
Fax: +34 93 316 09 01; Phone: +34 93 316 08 52
e-mail: paco.munoz@upf.edu

ABSTRACT

Stroke is an acute vascular event which obstructs the blood supply to the brain, leading to an ischaemic process affecting neurons, glial cells, and vessels. The identification of a biomarker for stroke is critical to ensure correct treatment which depends on a rapid and accurate diagnosis. We have measured the production of reactive oxygen species and NO by biochemical and confocal microscopy imaging techniques in cell lines exposed to ischaemic conditions and human brain from stroke patients. Since nitric oxide (NO) and superoxide anion reacts with NO to produce peroxynitrite which induces the nitrotyrosination of the proteins, the nitrotyrosination of tissue and circulating fibrinogen was also quantified from plasma samples of control individuals and patients suffering from different types of ischaemic stroke and from animal models of ischaemia. High levels of fibrinogen nitrotyrosination were detected as early as 3 hours after the initiation of ischaemic strokes (atherothrombotic, cardioembolic, lacunar, and undetermined). Nitrotyrosination of fibrinogen affected its structure and, interestingly, *in vitro* experiments showed that fibrinogen nitrotyrosination can be decreased by reduced glutathione, suggesting a possible therapeutic approach in stroke. Summarizing, quantification of nitrotyrosinated fibrinogen might be useful as an early plasmatic biomarker of stroke as well as a therapeutic target.

Key words: Stroke biomarker; nitric oxide; peroxynitrite; nitrotyrosination; fibrinogen.

Nonstandard abbreviations used: Ab, antibody; BSA, bovine serum albumin; BV2, mouse microglial cells; CP, cortical perfusion; DCF, dichlorofluorescein; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; GSH, reduced glutathione; HC-VSMC, human cerebral

vascular smooth myocytes; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; MCA, middle cerebral artery; MG, methylglyoxal; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; 3NT, 3-nitrotyrosine; O_2^-, superoxide anion; OGD, oxygen-glucose deprivation; $ONOO^-$, peroxynitrite anion; RT, room temperature; rt-PA, recombinant tissue plasminogen activator; SH-SY5Y, human neuroblastoma cells; SIN-1, 3-morpholinosydnonimine hydrochloride; SNP, sodium nitroprusside.

Introduction

Stroke is a leading cause of death and disability worldwide [1,2] and the currently available treatments targeting clot formation carry certain risks as they may increase the risk of bleeding if admininstration is delayed after stroke onset [3]. Thereby, the effectiveness of stroke treatment depends on the rapid and accurate diagnosis of stroke [4]. The identification of a biomarker of the early events involved in stroke pathophysiology would add to the clinical and neurological examination, being of invaluable help in tackling stroke management, and widely requested by the scientific and medical communities [3,5-7].

Stroke is an acute vascular event obstructing the blood supply to the brain, leading to an ischaemic process affecting neurons, glial cells, and vessels. Surrounding the ischaemic lesion is a penumbra area, where neurons are still alive, with a residual blood perfusion that allows inflammatory cell infiltration [8]. In this scenario, nitric oxide (NO), a molecule with pleiotropic effects in the brain [9], is increased to secure blood flow within the compromised area [10]. Following the ischaemic event there is also a burst in the production of free radicals such as the superoxide anion ($O_2^{\cdot-}$) [11,12]. NO reacts with $O_2^{\cdot-}$ producing the highly reactive peroxynitrite anion ($ONOO^-$) [13]. Peroxynitrite, among other harmful effects, nitrates proteins [14,15] via an irreversible chemical process consisting of the addition of a nitro group (NO_2) to tyrosine residues generating 3-nitrotyrosine (3NT). This process, also termed nitrotyrosination, is a post-translational modification that normally leads to a loss of protein function.

Fibrinogen is one of the most abundant plasma proteins with a physiological function in blood coagulation, but serves as a risk factor [16] as well as a prognosis factor [17,18] for stroke that contributes to exacerbation of brain damage [19]. Fibrinogen has been reported to be nitrotyrosinated in coronary artery disease, inducing a pro-thrombotic

status due to changes in the structure of the fibrin clots, which are more prone to produce microemboli [20].

In the present study, we have evaluated nitrotyrosination in brain tissues and circulating fibrinogen after ischaemic stroke.

Materials and methods

Cell culture and viability assay

Human neuroblastoma cells (SH-SY5Y), umbilical vein endothelial cells (HUVEC), cerebral vascular smooth myocytes (HC-VSMCs), and murine microglial cells (BV2) were cultured as previously described [14,21]. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method and expressed as a percentage of control [14]. The peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1; Sigma), the nitric oxide donor sodium nitroprusside (SNP; Sigma) and H_2O_2 were used to evaluate the impact of nitro-oxidative stress on cell viablility.

Immunodetection

SH-SY5Y cells (4 x 10^4 cells/well) were seeded on 1.5% gelatine-coated 12 mm coverslips. Cells were treated with SNP plus H_2O_2 (for 1 h) or SIN-1 (for 6 h). In other experiments, cells were challenged for 1 h with oxygen-glucose deprivation (OGD) and maintained in standard culture conditions for up to 24 h. Cells were fixed and incubated for 2 h at room temperature (RT) with 1:500 rabbit anti-nitrotyrosine polyclonal antibody (Molecular Probes) followed by incubation with 1:500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab (Dako) for 1 h at RT. Anti-neuronal NO synthase (nNOS), anti-inducible NOS (iNOS), and anti-tubulin polyclonal Abs (all from Santa Cruz Biotech), were used at 1:400 and 1:10 000 (for anti-tubulin) to detect different NOS in extracts from ipsilateral and

contralateral hemispheres of rat middle cerebral artery (MCA) occlusion models. Peroxidase-conjugated donkey anti-rabbit (Amersham Bioscience) was used as a secondary Ab at 1:5,000.

NO measurement

SH-SY5Y, BV2, HUVEC, and HC-VSMC cells were seeded on T-25 flasks (5 x 10^6 cells/flask) and subjected to OGD. Control media, 12 h post-ischaemic media and 24 h post-ischaemic media were collected and NO was measured using a nitrate/nitrite colorimetric assay kit (Cayman) [14].

Fibrinogen conformational state analysis by intrinsic fluorescence measurement

Intrinsic protein fluorescence emission is mainly due to tryptophan residues, which have a wavelength of maximum absorption of 280 nm and an emission peak ranging from 300 to 350 nm. Therefore the shift in protein fluorescence can be used to study the changes in protein conformational states [22]. Freeze-dried human fibrinogen (Sigma) was directly solubilized at 1 mg/mL in TBS (50 mM Tris base, 150 mM sodium chloride; pH 7.4). Fibrinogen was then incubated in the presence of 12 mM $CaCl_2$, with or without 100 μM SIN-1 at 37°C for 24 h in the dark. Intrinsic fluorescence measurements of these mixtures were determined in a Shimadzu spectrofluorophotometer (RF-5301). Samples were excited at 280 nm and fluorescence emission reading was recorded between 300 to 400 nm.

In vitro ischaemia

Human neuroblastoma cells (SH-SY5Y) were cultured as previously described [21]. Ischaemia was induced by OGD protocol. Briefly, the culture medium was replaced with a glucose-free balanced saline solution

containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 1.8 $CaCl_2$ and was placed in a nitrogen chamber for 1 h at 37ºC. Then, the OGD medium was replaced by culture medium and cells were incubated in standard culture conditions for up to 24 h after OGD. Cells were fixed and incubated for 2 h at RT with 1:500 rabbit anti-nitrotyrosine polyclonal Ab (Molecular Probes) or 1:200 mouse anti-methylglyoxal (MG) monoclonal Ab (NOF corporation) followed by incubation with 1:500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab (Dako) for 1 h at RT. In another set of experiments, to measure oxidative stress, cells were incubated with the fluorochrome 2,7-dichlorofluorescin diacetate (DCF-DA) at 5 µM 24 h after OGD.

Human brain samples

Human brain sections (5 µm) obtained from autopsies of patients who had a stroke were supplied by the Unitat d'Anatomia Patològica (Hospital del Mar). Brain sections were handled and exposed to primary (see previous section) and secondary Abs (1:5 000 biotinylated goat anti-mouse polyclonal Ab (Dako) as previously described) [15]. All images were taken with a Leica TCS SP confocal microscope and analysed with Leica confocal software (Leica).

Human plasma samples, provided by the Neurology Department (Hospital del Mar, Barcelona), were obtained from patients after atherothrombotic (17 individuals), cardioembolic (14 individuals), undetermined (8 individuals), and lacunar (12 individuals) strokes, and from controls (13 individuals). The stroke specialist in charge of the patients estimated that blood extraction was carried out approximately 3 h after ischaemic stroke. All procedures were approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra.

Focal cerebral ischaemia in rats

Focal cerebral ischaemia was produced by transient intraluminal occlusion of the MCA in Sprague-Dawley rats (3 months old), as previously reported [8]. Plasma (500 μL) was extracted from the rats after 3, 6, 12, and 24 h of MCA occlusion and analysis of fibrinogen nitrotyrosination was performed. The procedure was approved by the Ethics Committee of the Universitat de Barcelona.

Fibrinogen immunoprecipitation

To immunoprecipitate fibrinogen, 350 μL of human and rat plasma samples were exposed to 5 μg of anti-human fibrinogen polyclonal Ab (Dako) or to 5 μg of anti-rat fibrinogen polyclonal Ab (Accu-Specs). Samples were shaken overnight at 4ºC. Following addition of 20 μg of protein G-sepharose (Sigma), samples were shaken for 2 h at 25ºC. Samples were subsequently centrifuged at 10 000 rpm for 10 min and washed 3 times with phosphate-buffered saline (PBS). Immunoprecipitated proteins were separated from protein G and from the Ab by boiling samples at 100ºC for 6 min. Proteins were identified by western blot using 10% Tris-glycine gels. Primary anti-fibrinogen (1:1 000) and anti-nitrotyrosine (1:1 000) and secondary peroxidase-conjugated donkey anti-rabbit Ab (1:5 000; Amersham Bioscience) were used. Bands were visualized using Super Signal (Pierce) and Amersham Bioscience Hyperfilm ECL kit.

Quantitative 3NT analysis of plasmatic fibrinogen

Fibrinogen was immunoprecipitated from 350 μL of either human plasma or rat plasma. 3-Nitrotyrosine (3NT) is a stable product that can be determined spectrophotometrically by the absorbance measurement at 415 nm (pH > 9) [23]. A calibration curve using serial dilutions of free 3NT (Sigma) was used to quantify the nitrotyrosination of the fibrinogen samples (expressed as μg of 3NT/mL). To evaluate the effect of reduced

glutathione (GSH), inmmunoprecipitated fibrinogen was incubated for 20 min with either 500 μM pure peroxynitrite (Cayman) or 500 μM peroxynitrite plus 500 μM GSH (Sigma).

Transmission electron microscopy

Samples of 5 μL of fibrinogen (1 mg/mL) incubated in the absence or presence of 100 μM SIN-1 (a peroxynitrite donor) were applied to carbon-coated Formvar grids (Sigma). Samples were stained with 5 μL of 0.5% phosphotungstic acid solution (Sigma). Samples were examined under a JEOL 1200 EX II electron microscope at 80 kV.

Statistics

All data are expressed as mean ± SEM. Statistical analysis was performed with paired Student's *t*-test and non-parametric ANOVA (Kruskall Wallis Test).

Results

Ischaemic conditions induce nitro-oxidative stress in cell models and brains from stroke patients

Human brain slices obtained from autopsies of stroke patients (Fig. 1A) were analysed for free radical production through the presence of methylglyoxal (MG), a harmful intracellular by-product of oxidative stress which induces protein glycation (left panels), and nitrotyrosination secondary to peroxynitrite anion ($ONOO^-$) production (right panels). Positive staining for both MG and nitrotyrosination was colocalized within the infarcted area, identified post-mortem at the hospital pathology department. Unaffected areas located in the same brain slice (control) showed no signals of nitrotyrosination or glycation.

The impact of ischaemia on oxidative stress and nitrotyrosination of proteins was also evaluated in cell models. Human neuroblastoma cells were subjected to oxygen and glucose deprivation (OGD) for 1 h, followed by reoxygenation and maintenance in growth medium containing glucose for up to 24 h (Fig. 1B). This protocol mimicked the ischaemia-reperfusion process that occurs during stroke [24]. Following ischaemia, cells showed a burst in free radical production, measured by the oxidation of DCF (left panels), MG production (middle panels), and nitrotyrosination of proteins (right panels). The production of NO triggered by OGD (a task difficult to accomplish using the whole tissue) and the cytotoxic effects of peroxinitrite were further studied in different cell-line models (Fig. 2). After the ischaemic challenge neurons, microglia, and endothelial cells showed an accumulated increase in NO production that lasted for up to 24 h ($p < 0.05$). Endothelial cells and microglia were the highest NO producers under both basal and ischaemic conditions. Brain vascular myocytes did not show any effect on NO release due to ischaemia (Fig. 2A).

The cytotoxic effect of peroxynitrite was tested in the human neuroblastoma cell model. Sodium nitroprusside (SNP), an NO donor, was not found to be toxic at the highest concentration used (1.5 mM) (Fig. 2B). The combination of SNP with non-toxic concentrations of H_2O_2 (used as an oxidative stress source), induced neurotoxicity at 10 μM H_2O_2 ($p < 0.05$) even with the lowest SNP concentration (0.5 mM), which is closest to physiological concentrations obtained in an NO production burst [9]. These findings can be explained as a result of the peroxynitrite-induced neurotoxicity following peroxynitrite formation in the presence of SNP and H_2O_2 [9]. This possibility was evaluated directly using SIN-1, a peroxynitrite donor. Increasing concentrations of SIN-1 reduced cell viability in different cell types (Fig. 2C). The threshold at which cytotoxicity occured was 1 mM SIN-1 for endothelial and neuroblastoma

cells, whereas 100 μM of SIN-1 was required in the more susceptible myocytes and microglial cells (Fig. 2C).

Nitrotyrosination of proteins secondary to peroxynitrite production was tested by immunofluorescence microscopy on neuroblastoma cells treated with SNP + H_2O_2 (Fig. 3A) or SIN-1 (Fig. 3B). Control non-treated cells showed very low levels of nitrotyrosination, a mechanism that cells may use under normal metabolic conditions to label proteins for degradation by the proteosome [25]. On the other hand, treated cells presented significantly higher levels of nitrotyrosination. Together, these results demonstrate that ischaemic conditions – such as stroke – induced protein nitrotyrosination due to enhancement of the production of oxidative stress and NO, which in turn increased peroxynitrite levels.

Stroke induces nitrotyrosination of fibrinogen

Next, we evaluated the possibility that peroxynitrite could spread its harmful effects into neighbouring tissues, by looking specifically at the nitrotyrosination of plasma proteins. Fibrinogen was immunoprecipitated from human plasma belonging to a healthy control and a stroke patient (Fig. 4A) and nitrotyrosination was analyzed by western blot using anti-nitrotyrosine antibodies. The stroke patient presented higher levels of nitrotyrosinated fibrinogen compared to the control. The consequence of fibrinogen nitrotyrosination was evaluated *in vitro*. Electron microscopy (Fig. 4B) and intrinsic protein fluorescence (Figs 4C and 4D) showed that *in vitro* nitrotyrosination of fibrinogen affected its structure. Nitrotyrosinated fibrinogen formed globular structures clearly different to the needle-shaped geometry observed in non-treated fibrinogen (Fig. 4B). Another piece of evidence indicating a structural change in fibrinogen after nitrotyrosination was obtained by analyzing the shift in intrinsic

protein fluorescence that indicates a conformational change in proteins [22]. A reduction in the intrinsic fluorescence emission was detected in nitrotyrosinated fibrinogen ($p < 0.001$) (Figs 4C and 4D).

Due to the fact that a circulating protein like fibrinogen is nitrotyrosinated *in vitro*, and considering the high nitro-oxidative stress found in brain regions suffering a stroke, we decided to evaluate fibrinogen nitrotyrosination in plasma as a biomarker for stroke. Quantification of fibrinogen nitrotyrosination was evaluated both in an *in vivo* animal model of brain ischaemia and in patients diagnosed with different types of ischaemic stroke.

Occlusion of the MCA in rats (Fig. 5) produced an infarction affecting the ipsilateral cortex and striatum. The mean ± SD infarct volume was 315.9 ± 64.4 mm^3, measured as previously described [26]. Our own data and previous reports [9] have shown increased NO production following a stroke, most likely as a consequence of augmented expression of NOS. An induction in iNOS protein expression was obtained only in the ipsilateral ischaemic hemisphere 24 h after ischaemia (Fig. 5A), without major changes in the expression of nNOS protein.

Plasma samples were extracted from rats before the experimental ischaemia was triggered (0 h) and at 3, 6, 12, and 24 h after the occlusion. A large increase in plasma fibrinogen nitrotyrosination with a peak at 3 h was measured. Elevated levels of nitrotyrosination were detected up to 24 h after MCA occlusion (Fig. 5B) demonstrating that fibrinogen nitrotyrosination was maintained as a consequence of brain ischaemia.

Similar to the brain ischaemia animal model, human nitrotyrosinated fibrinogen was detected in the plasma of patients suffering a stroke (Fig. 6A). The presence of nitrotyrosinated fibrinogen was measured from 3–6 h after suffering different types of ischaemic stroke (diagnosed at the Neurology Department of the Hospital del Mar, Barcelona). The levels of nitrotyrosinated fibrinogen (3NT-fibrinogen)

from healthy controls were significantly lower than in ischaemic stroke patients: atherothrombotic ($p < 0.005$), cardioembolic ($p < 0.01$), undetermined ($p < 0.005$), and lacunar ($p < 0.01$) strokes.

GSH reduces fibrinogen nitrotyrosination in vitro

Finally, we evaluated whether the use of antioxidants could decrease the nitrotyrosination of circulating fibrinogen. Fibrinogen was immunoprecipitated from control human plasma samples and later treated with pure peroxynitrite in the presence (500 μM) or absence of the antioxidant GSH (Fig. 6B). Interestingly, GSH significantly reduced fibrinogen nitrotyrosination.

Discussion

The effects of ischaemic stroke largely depend on the localization and severity of the infracted region of the brain. While there are often clinical clues guiding doctors towards a correct diagnosis, these features may be absent or even mistaken by other stroke "mimic" conditions. Late diagnosis of an ischaemic stroke results in substantial neurological deficits. Therefore, rapid and accurate diagnosis of ischaemic stroke would reduce mortality and systemic damage associated with late diagnosis.

Under ischaemic conditions such as in a stroke, the tissue reacts with a cascade of events which aim to protect the damaged brain, e.g. increased NO production to vasodilate and maintain blood perfusion [27]. However, ischaemia also results in mitochondrial dysfunction, which in turn generates a burst in free radical production [11] and the generation of peroxynitrite [28]. Subsequent protein nitrotyrosination, which occurs during, and participates in, massive tissue parenchyma damage, is largely

responsible for cell death [9]. It is this event precisely – protein nitrotyrosination – on which we have based our search for a novel biomarker for ischaemic stroke.

Our findings suggest that the toxic effects of peroxynitrite are not merely confined to brain tissues affected by the disrutption in blood supply, but they can target plasma circulating proteins as well. We detected fibrinogen nitrotyrosination as early as 3 h after the first stroke symptoms. Therefore, the profile of fibrinogen nitrotyrosination after brain ischaemia seems to be a feasible indicator of the pathophysiological events taking place at the ischaemic focus. Interestingly all the subtypes of ischaemic strokes (atherothrombotic, cardioembolic, undetermined, and lacunar) show significantly high values of fibrinogen nitrotyrosination, suggesting that regardless of the causes of the ischaemic stroke, shared peroxynitrite production processes are involved. Absolute increases in nitrotyrosinated fibrinogen levels have been reported in stroke patients, probably reflecting its up-regulation as an acute phase reactive protein [29], and have been related to both prognosis and risk factors of stroke [16,17].

Moreover, we have found that fibrinogen nitrotyrosination changes the structure of the molecule. These conformational changes affect clot formation, with micro-emboli more prone to be generated [20]. We have also demonstrated *in vitro* the usefulness of GSH, a well-known physiological antioxidant [30], to prevent fibrinogen nitrotyrosination, thereby, presenting the possibility for the early use of antioxidants in the treatment of stroke.

In summary, in animal models of brain ischaemia and in human stroke patients, the presence of nitrotyrosinated fibrinogen is significantly higher than in controls. Determination of nitrotyrosinated fibrinogen complies with several criteria for an ideal biomarker of stroke: biological plausibility, a plasma biomarker, and rapidity of putative testing methods. One possible limitation of our study is that fibrinogen nitrotyrosination also occurs in cardiac ischaemia. However, both clinical and analytical

factors clearly distinguish these conditions allowing a rapid action in cardiac ischaemia without any plasmatic determination. Further work should be carried out to demonstrate the role of the nitrotyrosination of fibrinogen in the progression of the stroke and the neurological damages associated.

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Conflict of interest statement

The authors of the present work declare that they do not have any conflict of interest for the publication of this work.

FIGURE 1

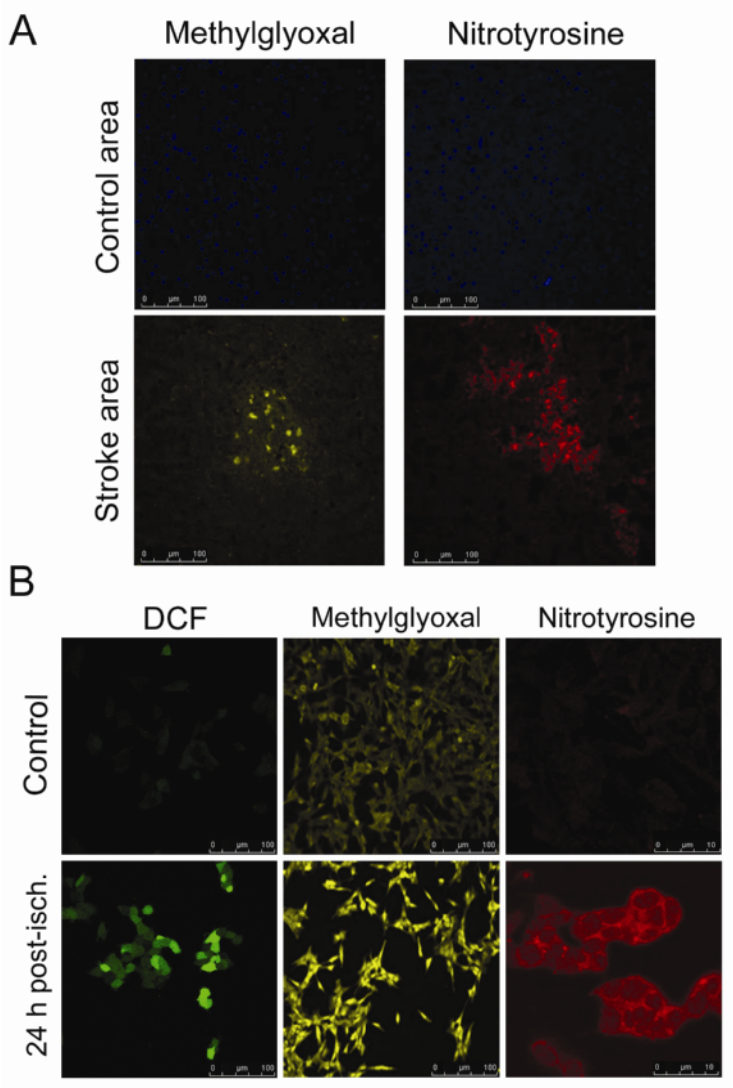


Figure 1 Ischaemia induces nitro-oxidative stress. (**A**) Immunofluorescence staining of MG and nitrotyrosine in brain slices obtained a stroke patient. Immunofluorescence images using anti-nitrotyrosine Ab (red) and anti- MG ab (yellow) appeared in the infarcted area (lower panels). The unaffected (control) area was negative for both MG and nitrotyrosine(upper panels). Nuclei are stained in blue. (**B**) Human neuroblastoma cells were subjected to control (top) or *in vitro* ischaemia conditions and later re-perfused with normal medium for up to 24 h (bottom). Images show free radical production detected by DCF fluorescence (green), MG (yellow), and nitrotyrosination (red). DCF = dichlorofluorescein; MG = methylglyoxal.

FIGURE 2

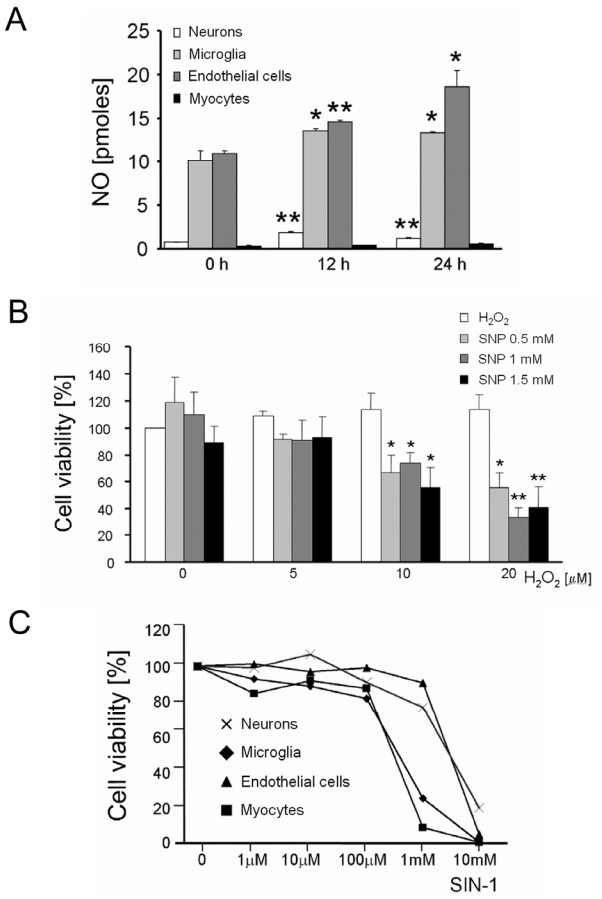


Figure 2 Peroxynitrite induces cell death in neurons, microglia, and vascular cells. (**A**) NO production was measured in neurons, microglia, and endothelial and brain vascular smooth muscle cell (myocyte) cultures challenged with *in vitro* ischaemia and reoxygenated with growing medium at 0, 12, and 24 h. Data are mean ± SEM values of 4 separate experiments. *$p < 0.05$; **$p < 0.005$ in comparison to basal conditions (0 h) by paired Student's *t*-test. (**B**) Human neuroblastoma cells were treated with increasing concentrations of SNP and H_2O_2. Cells were incubated for 6 h and cell viability was assayed by MTT reduction. Data are expressed as a percentage of control cells. Data are mean ± SEM values of 7 experiments performed by tripliclate. *$p < 0.05$; **$p < 0.005$ in comparison to basal conditions (0 μM H_2O_2) by paired Student's *t*-test. (**C**) Cells were treated for 24 h with increasing concentrations of SIN-1 (peroxynitrite donor). Data are mean values of 4–6 independent experiments performed by triplicate. SIN-1 = 3-morpholinosydnonimine hydrochloride; SNP = sodium nitroprusside.

FIGURE 3

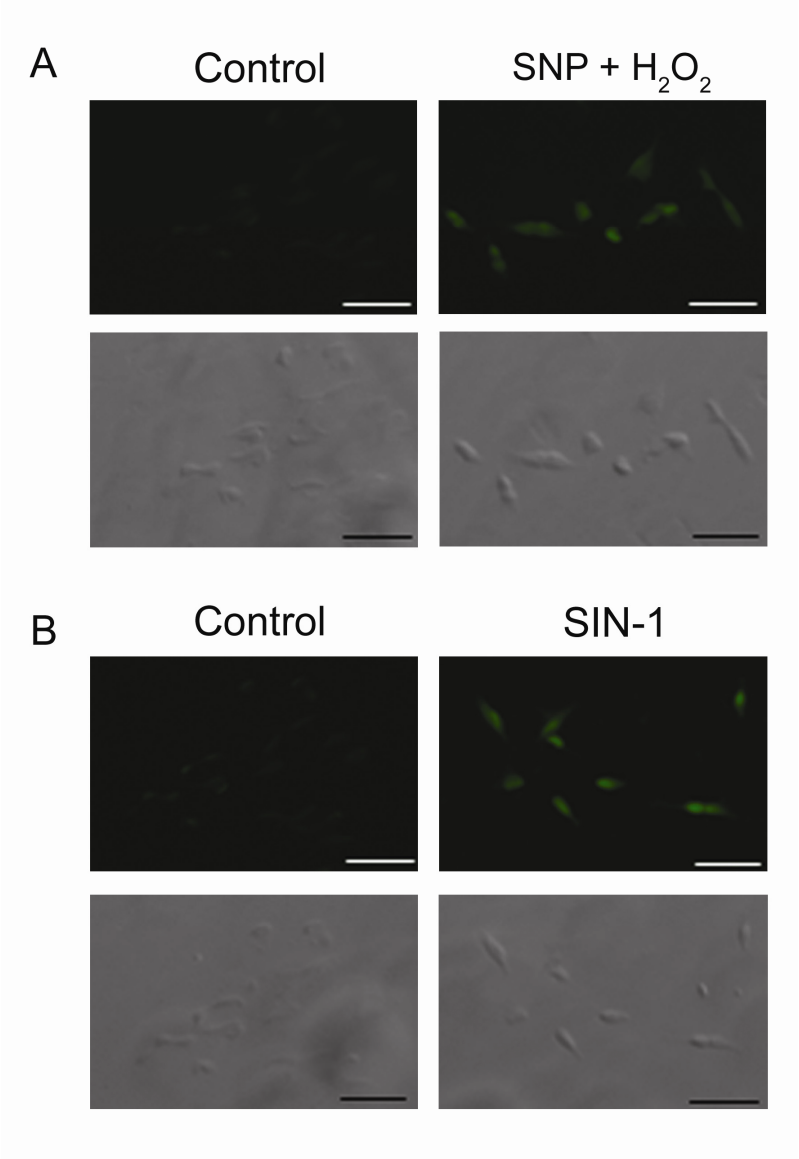


Figure 3 Peroxynitrite induces protein nitrotyrosination. SH-SY5Y cells were treated with 1.5 mM SNP plus 20 μM H_2O_2 for 1 h (**A**) or 500 μM SIN-1 for 6 h (**B**). Protein nitrotyrosination in neuronal cells is shown by immunofluorescence analysis incubating with an anti-3NT Ab. SH-SY5Y = human neuroblastoma cells; SIN-1 = 3-morpholinosydnonimine hydrochloride; SNP = sodium nitroprusside.

FIGURE 4

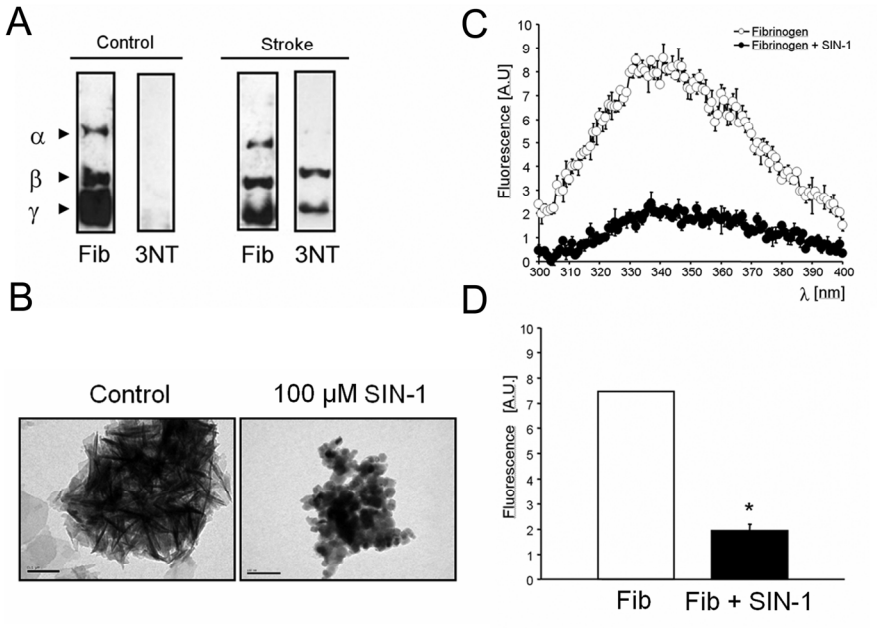


Figure 4 Structural changes in nitrotyrosinated fibrinogen. (**A**) Representative western blots showing the extent of nitrotyrosination in fibrinogen immunoprecipitated from plasma samples of a healthy control (left panels) and a stroke patient (right panels). Membranes were revealed using both an anti-fibrinogen antibody (Fib) and an anti-nitrotyrosine antibody (3NT). The α, ß, and γ bands show the different fibrinogen chains. (**B**) Electron micrographs of fibrinogen incubated without or with 100 µM SIN-1 at 37°C for 24 h in the dark. Samples were negatively-stained and analysed by TEM. Bars represent 100 nm. (**C**) Intrinsic fluorescence emission of nitrotyrosinated fibrinogen. 1 mg/mL human fibrinogen was incubated with or without 100 µM SIN-1 at 37°C for 24 h in the dark. Intrinsic fluorescence measurements (Ex: 280 nm; Em: 300–400 nm) were performed in triplicates. Data are mean ± SEM. (**D**) Graph shows the intrinsic fibrinogen fluorescent signal recorded at 340 nm. Bars are mean ± SEM of 4 independent experiments performed in triplicate. *$p < 0.001$ by paired Student's *t*-test. SIN-1 = 3-morpholinosydnonimine hydrochloride; TEM = transmission electron microscopy;

FIGURE 5

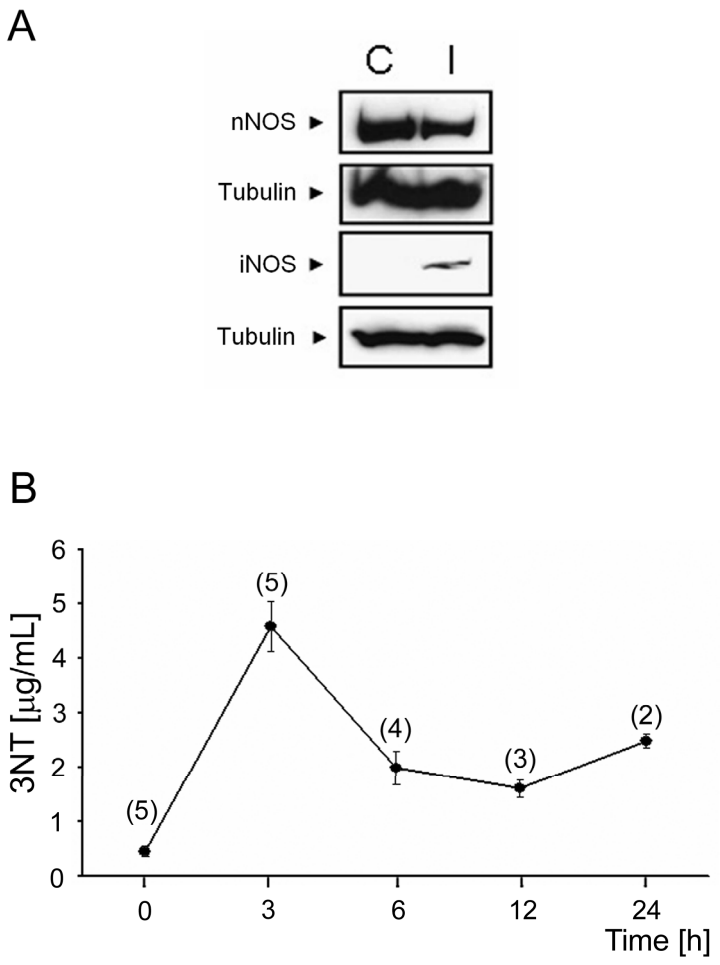


Figure 5 *In vivo* ischaemia causes fibrinogen nitrotyrosination in a rat model. (**A**) NOS expression in brains of animal models of ischaemia. nNOS and iNOS expression were studied in the ipsilateral (I) and contralateral (C) part of the rat brain 24 h after ischaemia. (**B**) A total of 19 rats were subjected to MCA occlusion. Rat plasma samples were obtained at 0, 3, 6, 12, and 24 h post-ischaemia. Fibrinogen nitrotyrosination levels were determined by immunoprecipitating fibrinogen and measuring its absorbance at 415 nm. Data are expressed as the mean ± SEM. The number of samples analyzed at each time is indicated in parentheses. MCA = middle cerebral artery; iNOS = inducible nitric oxide synthase; nNOS = neuronal NOS.

FIGURE 6

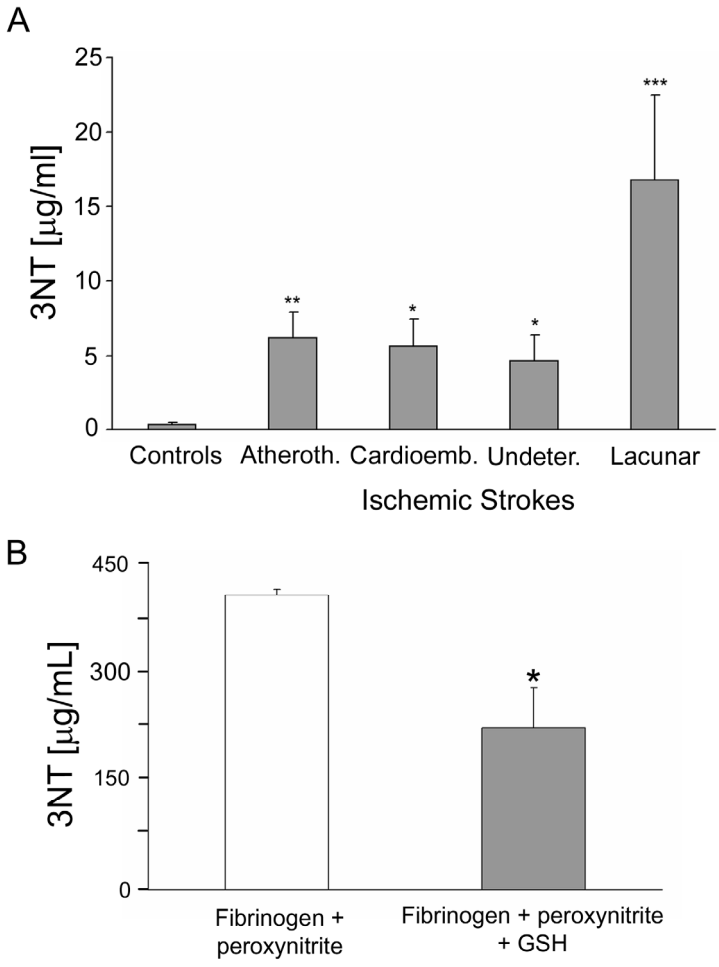


Figure 6 Plasmatic fibrinogen is nitrotyrosinated in stroke patients. (**A)** Quantification of plasma fibrinogen nitrotyrosination in controls (n = 23) and atherothrombotic (n = 17), cardioembolic (n = 14), undetermined (n = 9) and lacunar (n = 12) stroke patients. Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***p < 0.001; by non-parametric ANOVA (Kruskall Wallis Test). (**B**) GSH inhibits the peroxynitrite-induced fibrinogen nitrotyrosination. Human plasma fibrinogen immunoprecipitated from a healthy donor was incubated with 500 µM pure peroxynitrite for 20 min in the absence or presence of 500 µM GSH. *$p <$ 0.005 by paired Student's *t*-test. GSH = reduced gluthathione.

References

1. Ingall, T. Stroke--incidence, mortality, morbidity and risk. *J. Insur. Med.* 36, 143-152 (2004).

2. Feigin, V. L., Lawes, C. M., Bennett, D. A., Barker-Collo, S. L. & Parag, V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *Lancet Neurol.* 8, 355-369 (2009).

3. Chavez, J. C., Hurko, O., Barone, F. C. & Feuerstein, G. Z. Pharmacologic interventions for stroke: looking beyond the thrombolysis time window into the penumbra with biomarkers, not a stopwatch. *Stroke* 40, e558-e563 (2009).

4. Kennedy, J. *et al.* Fast assessment of stroke and transient ischaemic attack to prevent early recurrence (FASTER): a randomised controlled pilot trial. *Lancet Neurol.* 6, 961-969 (2007).

5. Goldstein, L. B. & Simel, D. L. Is this patient having a stroke? *JAMA* 293, 2391-2402 (2005).

6. Yu, E. H., Lungu, C., Kanner, R. M. & Libman, R. B. The use of diagnostic tests in patients with acute ischemic stroke. *J. Stroke Cerebrovasc. Dis.* 18, 178-184 (2009).

7. Laskowitz, D. T., Kasner, S. E., Saver, J., Remmel, K. S. & Jauch, E. C. Clinical usefulness of a biomarker-based diagnostic test for acute stroke: the Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study. *Stroke* 40, 77-85 (2009).

8. Ferrer, I. & Planas, A. M. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J. Neuropathol. Exp. Neurol.* 62, 329-339 (2003).

9. Guix, F. X., Uribesalgo, I., Coma, M. & Munoz, F. J. The physiology and pathophysiology of nitric oxide in the brain. *Prog. Neurobiol.* 76, 126-152 (2005).

10. Zhu, D. Y. *et al.* Inducible nitric oxide synthase expression in the ischemic core and penumbra after transient focal cerebral ischemia in mice. *Life Sci.* 71, 1985-1996 (2002).

11. Cuzzocrea, S., Riley, D. P., Caputi, A. P. & Salvemini, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol. Rev.* 53, 135-159 (2001).

12. Niizuma, K., Endo, H. & Chan, P. H. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J. Neurochem.* 109 Suppl 1, 133-138 (2009).

13. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A* 87, 1620-1624 (1990).

14. Coma, M. *et al.* Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. *Brain* 128, 1613-1621 (2005).

15. Guix, F. X. *et al.* Amyloid-dependent triosephosphate isomerase nitrotyrosination induces glycation and tau fibrillation. *Brain* 132, 1335-1345 (2009).

16. Rudnicka, A. R., Mt-Isa, S. & Meade, T. W. Associations of plasma fibrinogen and factor VII clotting activity with coronary heart disease and stroke: prospective cohort study from the screening phase of the Thrombosis Prevention Trial. *J. Thromb. Haemost.* 4, 2405-2410 (2006).

17. del Zoppo, G. J. *et al.* Hyperfibrinogenemia and functional outcome from acute ischemic stroke. *Stroke* 40, 1687-1691 (2009).

18. Di, N. M. & Singh, P. Is plasma fibrinogen useful in evaluating ischemic stroke patients?: why, how, and when. *Stroke* 40, 1549-1552 (2009).

19. Wardlaw, J. M., Zoppo, G., Yamaguchi, T. & Berge, E. Thrombolysis for acute ischaemic stroke. *Cochrane. Database. Syst. Rev.* CD000213 (2003).

20. Vadseth, C. *et al.* Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J. Biol. Chem.* 279, 8820-8826 (2004).

21. Coma, M. *et al.* Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells. *Neurobiol. Aging* 29, 969-980 (2008).

22. Vivian, J. T. & Callis, P. R. Mechanisms of tryptophan fluorescence shifts in proteins. *Biophys. J.* 80, 2093-2109 (2001).

23. De Filippis, V., Frasson, R. & Fontana, A. 3-Nitrotyrosine as a spectroscopic probe for investigating protein protein interactions. *Protein Sci.* 15, 976-986 (2006).

24. Xu, J. *et al.* Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells. *Stroke* 31, 1744-1751 (2000).

25. Amici, M., Lupidi, G., Angeletti, M., Fioretti, E. & Eleuteri, A. M. Peroxynitrite-induced oxidation and its effects on isolated proteasomal systems. *Free Radic. Biol. Med.* 34, 987-996 (2003).

26. Justicia, C. *et al.* Anti-VCAM-1 antibodies did not protect against ischemic damage either in rats or in mice. *J. Cereb. Blood Flow Metab* 26, 421-432 (2006).

27. Moro, M. A., Cardenas, A., Hurtado, O., Leza, J. C. & Lizasoain, I. Role of nitric oxide after brain ischaemia. *Cell Calcium* 36, 265-275 (2004).

28. Taffi, R. *et al.* Plasma levels of nitric oxide and stroke outcome. *J. Neurol.* 255, 94-98 (2008).

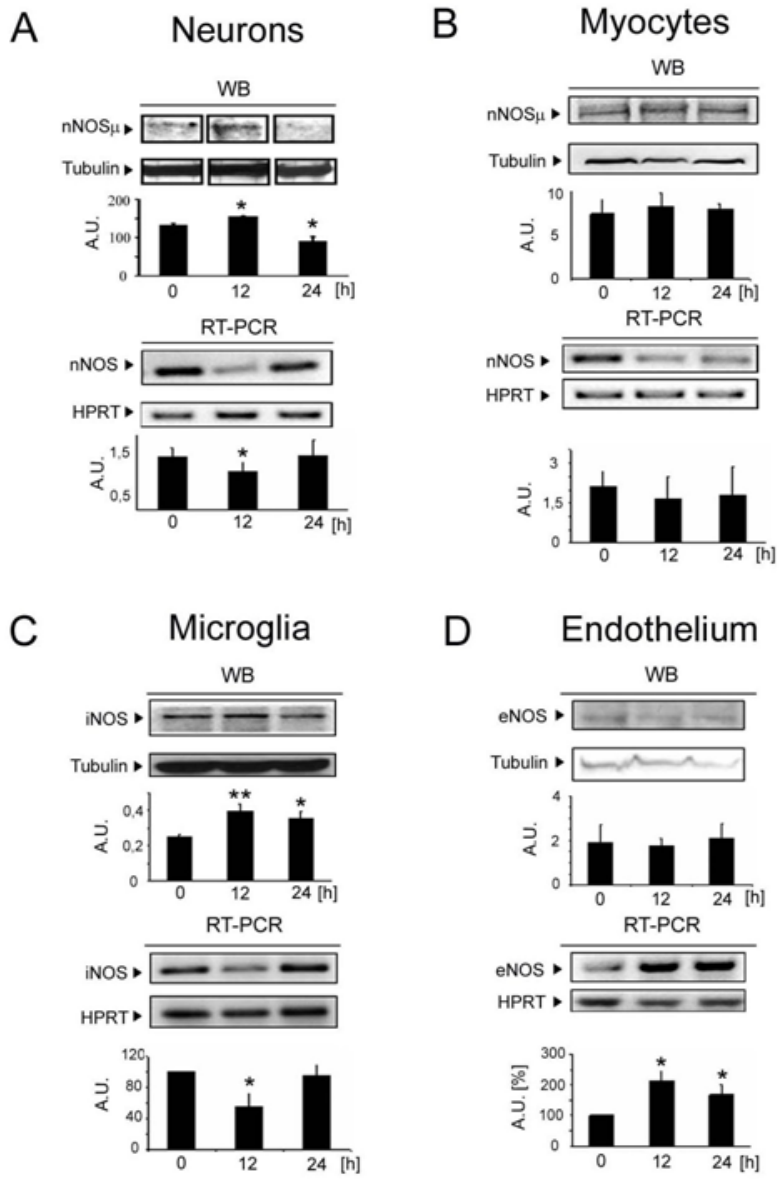
29. Dziedzic, T. Clinical significance of acute phase reaction in stroke patients. *Front Biosci.* 13, 2922-2927 (2008).

30. Ozkul, A. *et al.* Oxidative stress in acute ischemic stroke. *J. Clin. Neurosci.* 14, 1062-1066 (2007).

CHAPTER V

OTHER RESULTS

Modulation of nitric oxide syntase mRNA and protein levels in response to in vitro ischemia.



Ischemic insult affects the expression of the different NOS

NO is produced by the different NOS. For this reason we examined the effect that ischemia has on the mRNA and protein levels of nNOS, eNOS and iNOS. We used different cell lineages that were subjected to OGD for 1 hour and later reperfused with normal growing medium for 12 and 24 hours respectively.

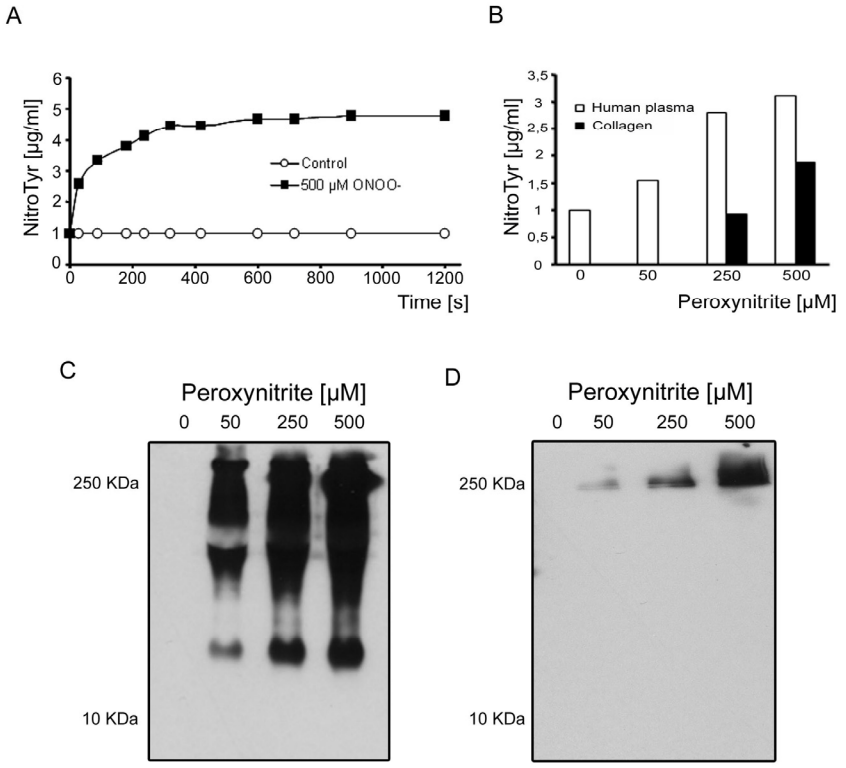
In human neuroblastoma cells we addressed the study of nNOS (A). We detected an early increase in nNOS protein 12 h after ischemia. At that time a lower mRNA transcription was observed ($p<0.05$) correlating with the decrease in protein obtained at 24 h ($p<0.05$). 24 h after ischemic challenge the mRNA transcription levels for nNOS were recovered.

The expression of iNOS has been reported in systemic rat vascular smooth muscle cells (Guix *et al.* 2005) so we decided to study if ischemia was able to modulate the expression of iNOS in human brain vascular smooth muscle cells (HC-VSMC). Neither iNOS protein nor iNOS mRNA were detected in HC-VSMC (data not shown). Interestingly, when we repeated the analysis in HC-VSMC directed to nNOS instead of iNOS, we obtained a positive result (B). nNOS protein is constitutively present in cerebral vascular myocytes and its expression is not affected by *in vitro* ischemia as well as the nNOS mRNA levels.

Microglial cells have been classically pointed as the main responsible for the NO burst occurring after brain ischemia. For this reason we studied the pattern of iNOS expression in microglial cells (BV2 mouse cell line) challenged with *in vitro* ischemia (C). Firstly we found that iNOS is present in resting control microglial cells although there was a significant increase in its expression 12 hours after the ischemic challenge. It was maintained even 24 hours later. The analysis of iNOS mRNA revealed, once again, a drop in the iNOS mRNA levels at 12h that was normalized 24h after the ischemic insult.

We did not find any significant change in eNOS protein after endothelial cells (HUVEC) were subjected to *in vitro* ischemia, but eNOS mRNA levels become clearly upregulated after ischemia (D).

Peroxynitrite induces the nitrotyrosination of plasmatic proteins



Stroke induces the nitrotyrosination of plasmatic proteins

Peroxynitrite is a short lived oxidant but its capability to diffuse through biological membranes can spread its harmful effects into neighbouring cells and tissues. In ischemia-reperfusion processes there is an interaction between blood plasma and the ischemic brain lesion where peroxynitrite is

being produced. Plasma is known to have antioxidant molecules and free radical scavengers where even albumin is assuming an antioxidant role. Therefore we addressed the study of peroxynitrite activity in total plasma. A plasma sample from a healthy donor was incubated *in vitro* with 500 μM peroxynitrite (A). A dramatic increase in nitrotyrosine formation was obtained indicating that plasmatic proteins can be easily nitrotyrosinated. Moreover, nitrotyrosination extent revealed to be a peroxynitrite concentration dependent process (B, C, D) as we demonstrated testing both total plasma and a particular circulating protein (collagen) with increasing concentrations of peroxynitrite.

Material and Methods

In vitro ischemia

Ischaemia was induced by an oxygen-glucose deprivation (OGD) protocol. Briefly, the culture medium was replaced with a glucose-free balanced saline solution containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 1.8 $CaCl_2$ and was placed in a nitrogen chamber for 1 h at 37ºC. Then, the OGD medium was replaced by culture medium and cells were incubated in standard culture conditions for up to 24 h after OGD.

Protein identification by Western Blot

Sample brains or cell cultures were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiotreitol, pH 7.4 and a protease inhibitor cocktail (Roche). Protein concentration was determined by a Bio-Rad protein assay. Protein samples were analyzed by using 10% Tris-glycine gels for fibrinogen nitrotyrosination detection or by using 3-8% Tris-acetate gels (Invitrogen) for NOS detection. Gels were ran at 150 V for 1

h and transferred to nitrocellulose membranes (Millipore) at 100 V for 2 h. Membranes were blocked in Tween 20-Tris buffer solution (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), containing 5% milk, and incubated for 2 h at 25ºC with 1:1,000 rabbit anti-nitrotyrosine polyclonal Ab (Invitrogen) or rabbit polyclonal anti-fibrinogen Ab (Dako). In the case of anti-nNOS, anti-iNOS and anti-eNOS (all from Santa Cruz Biotech.) Abs, the membranes were blocked in Tween 20-Tris buffer solution (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), containing 3% BSA, and incubated for 2 h at 25ºC with 1:400 of primary Ab. Peroxidase-conjugated donkey anti-rabbit and anti-mouse Abs (Amersham Bioscience.) were used as secondary Abs at 1:5,000 for 1 h at 25ºC. Bands were visualized with Super Signal (Pierce) and Amersham Bioscience Hyperfilm ECL kit.

RNA isolation and RT-PCR analysis.

Total RNA from cell cultures was isolated using Trizol reagent (Invitrogen), following the manufacture's instructions. 1 μg of RNA was used in a reverse transcriptase-polymerase chain reaction (RT-PCR) using the OneStep RT-PCR Kit (Qiagen, Hamburg Germany). iNOS specific primerS (5'-CCATCACTGTGTTCCCCC-3' and 5'AAGGTGGCAGCATCCCC-3'), eNOS specic primers (5'-CAAGTATGCCACCAACCGGG-3' and 5'-ACTGAAGGGGGCTGCGG-3') and nNOS specific primers (5'-GAGAAGGAGCAGGGGGGG-3' and 5'-CACATTGGCTGGGTCCCC-3') were used for the amplification of the three different NOS isoforms. Hypoxanthine phosphoribosyltransferase (hPRT) (primers 5′-GGCCAGACTTTGTTGGATTTG-3′ and 5′-TGCGCTCATCTTAGGCTTTGT-3′) was used as positive control. Negative control was performed in the absence of oligonucleotide primers. Results were analyzed with Image Gauge software (Fuji Photo Film Co., Tokyo).

Peroxynitrite treatments

For the analysis of total plasma nitrotyrosination levels, plasma samples from healthy human donors were incubated with 500 μM peroxynitrite or PBS (controls) during 20 minutes. The nitrotyrosination of the samples was followed by the measurement of the absorbance at 415 nm. A dose-absorbance curve was performed with free 3-nitrotyrosine. Data was expressed as μg/mL of nitrotyrosine.

For the comparision of nitrotyrosination between total plasma and human collagen, 1,25 μg/mL of plasma samples from healthy human donors and rat collagen were incubated with PBS or increasing concentrations of peroxynitrite during 5 minutes. A dose-absorbance curve was performed with free 3-nitrotyrosine and the data was expressed as μg/mL of nitrotyrosine.

The dose-response curve of total plasma nitrotyrosination in response to increasing concentrations of peroxynitrite was assayed by incubating 1,25 μg/mL of plasma samples from healthy human donors with PBS or increasing concentrations of peroxynitrite during 5 minutes. A western blot was carried out with anti-nitrotyrosine antibody (1:1000).

The dose-response curve of rat collagen nitrotyrosination in response to increasing concentrations of peroxynitrite was assayed by incubating 1,25 μg/mL of rat collagen with PBS or increasing concentrations of peroxynitrite during 5 minutes. A western blot was carried out with anti-nitrotyrosine (1:1000) antibody.

4. Discussion

The physiological activity of the Glu/NMDAR/NO pathway has a relevant role in memory formation and neuronal plasticity [70-74]. However, superoxide anion reacts with high affinity with NO, producing the harmful peroxynitrite anion. This implies that NO turns toxic when it is produced under conditions of oxidative stress. In pathological events involving oxidative stress such as Alzheimer's disease or brain ischemia, two parallel events take place in relation with NO. Firstly, there is a loss in NO bioavailability and its physiological effects in memory formation and neuronal plasticity are lost. Secondly, peroxynitrite triggers the initiation of a harmful nitrative cascade.

The involvement of NO in AD pathology is based on the above mentioned pathological properties when NO is produced in an oxidative stress environment. There are solid evidences relating AD pathophysiology with oxidative stress [152,153]. As a matter of fact, Aß fibrils produce hydrogen peroxide [155-157]. In such oxidative circumstances caused by Aß fibrils, endogenous NO can be scavenged by superoxide anion, reducing NO bioavailability and promoting nitrative damage through the deleterious actions of peroxynitrite. In fact, specific cerebral regions in AD patients have higher protein nitrotyrosination levels [167,168] and important proteins related to neurotransmission secretion[170], glucose metabolism and cytoeskeleton[63,170,171] have also been found to be nitrotyrosinated in AD.

Brain ischemia is also a pathology in which oxidative stress plays an important role. Neurons are strictly aerobic cells whose metabolism depends entirely on an uninterrupted oxygen supply by an adequate blood perfusion. As a consequence, when the blood flow is interrupted by an ischemic event, ATP levels drop dramatically in the ischemic neurons permitting a massive calcium influx and the subsequent over release of the excitatory neurotransmitter glutamate[180], that may reach 50 times their normal levels, overexciting ischemic cells to death[192]. Glutamate is considered the major harmful agent after stroke since it induces directly

excitotoxicity [192]. Interestingly, NO also plays a key role in the neurotoxicity occurring after brain ischemia. Glutamate activates the NMDAR, producing a calcium influx that activates the nNOS, which is physically linked with NMDAR, producing a release of NO. Despite the protective effects that NO has when it is produced by the eNOS to promote vasodilatation in an attempt to recover blood flow after ischemia, the NO resulting from the overexcitation of the Glu/NMDAR/nNOS system has deleterious effects [216-218] as it has been shown experimentally using NOS inhibitors and by KO approaches[194 195-198]. In conclusion, when NO is overproduced in the context of ischemia it reacts with superoxide anion resulting in NO bioavailability loss due to the formation of peroxynitrite that will mediate cellular nitrative damage.

Alzheimer's disease: the disease of the vanishing memory

AD is a devastating neurodegenerative disease that affects millions of people worldwide. Moreover, the prevalence of the disease will increase in the following decades as a consequence of the general aging of the population. In this scenario, the primary human attitude, both in the medical care and in the biomedical research fields is to make an effort in order to ameliorate the life quality of patients and their families. However, AD offers us indirectly an excellent opportunity for the understanding of the biological counterpart of this complex phenomenon called *memory*. The known molecular actors in AD pathology can be considered memory supporting elements, which have lost their physiological regulation. Thus the progressive understanding of AD pathology might accompany a progressive comprehension of the biological processes supporting memory formation in the hippocampus. In this regard, my personal opinion is that definitive progress in AD therapy can only be achieved

together with a substantial progress in the understanding of memory formation.

The amyloid hypothesis

The amyoloidogenic processing of APP was discovered for its implication in AD pathology. There are convincing genetic evidences that demonstrate the relevance of Aß in AD pathology. At first, individuals with Down syndrome invariably develop AD since the amyloid precursor protein (APP) is codified in chromosome 21[89]. Secondly, mutations in APP[90-92] or presenilins (PS1, PS2) [93-95] are causative of the early onset of AD. Futhermore, a wide number of publications report on the harmful effect of Aß *in vitro* [219,220] and *in vivo* [221,222]. The use of transgenic mice overexpressing APP and PS1 also has contributed to settle the amyloid hypothesis [221-223]. But there are weak points in the amyloid hypothesis. The major objection to amyloid hypothesis is based in the lack of correlation between senile plaques and cognitive impairment which was raised by early works in the 90' [224]. Later, with the discovery of the undetectable oligomers [225] was proposed that the major damage was produced by these small amyloid aggregates [226]. In spite of the wide damage induced by the oligomers, there are controversial results in the dynamic of the senile plaques even in transgenic mice overexpressing APP and PS1 and in human PET studies [227].

APP and memory

Neuronal plasticity changes are necessary for memory formation. This plasticity has been well described at the level of synaptic strength modulation, but it also would apply at the level of neuron's structure.

Neurons are large cells with a complex morphology and a non-static architecture. Their morphology undergoes a continuous structural rearrangement. This dynamism is necessary to support complex performances such as memory and learning. To achieve this structural plasticity, the adhesion of the neurons to the extracellular matrix must be flexible and responsive to the synaptic activity of neuron's terminals. In that regard, the regulated APP cleavage might be a way of modulating the adhesion force of neuronal terminals to the extracellular matrix, in a necessary process to enable the structural rearrangements required in learning and memory. Although APP was identified as the amyloid precursor protein 23 years ago [85,86,88,228], we still do not fully understand its biological function in the central nervous system. The main obstacle to unveil APP function in neurons is that two homologues of APP exist (APLP1 and APLP2)[229] and can compensate for its function, whereas the triple knock out mice has a lethal phenotype[230] and can only be generated via complex breeding schemes. There are evidences that APP participates in the regulation of actin-based motility, is localized in the growth cones of neurons[231], promotes neurite outgrowth[232] and it is involved in processes of synapse formation[233,234] and in neuronal migration [235]. In addition, mice lacking the APP gene present age-related cognitive deficits and a reduction in LTP and synaptic markers [236]. Nevertheless, the most deeply justified evidences, supported with evolutionary data, confers APP a cell adhesion role [105,106].

It is logical to think that a defined biological system, such as the sequential APP cleavage by distinct proteases, cannot exist *only* in the context of pathology but it must be at least reflecting a loss in physiological activity. Therefore, the AD causative mutations in presenilins and APP genes might be reflecting a loss in neuron ability to modulate its adhesion force to the extracellular matrix, and as a consequence, a loss in neuronal plasticity. Following this reasoning, it can

be speculated that individuals with Down's syndrome have an abnormally enhanced adhesion force of its neuronal terminals to the extracellular matrix as they have an extra dose of APP gene. This would lead to an enhanced resistance to structural rearrangements and as a consequence, a decrease in neuronal plasticity. Indeed, individuals with Down's syndrome present senile plaques at early ages as a direct consequence of the excessive load of APP gene.

Therefore, one can conclude that APP cleavage is crucial for the physiology of memory, as mutations in genes that are associated with Aß production impair proper memory formation[90-92]. Instead of considering the amyloidogenic cascade as the epicenter of AD pathology it can also be interpreted as the pathological ending of the complex system supporting memory.

BACE1: a protease with a role in memory formation

BACE1 mRNA translation is tightly controlled by its 5'UTR [132-135]. It implies that the biological function of BACE1 must be tightly regulated. Interestingly, a close analysis reveals synaptic plasticity deficits and cognitive and emotional behavioural impairments in the *BACE1*-KO mice [139,140]. Another study demonstrates that BACE1 activity is essential for the APP-mediated enhancement of memory and activity-dependent synaptic plasticity [141]. These findings, together with the fact that synaptic activity directly modulates the levels of extracellular Aß [237], led us to hypothesize that a tightly regulated control of BACE1 activity might be crucial for the understanding of the simultaneous implication of BACE1 in synaptic plasticity and amyloid pathology —when this physiological regulation became disrupted by any source of neuronal stress.

Nitric Oxide: the physiological activator of the amyloidogenic APP cleavage

There are evidences suggesting a role of NO in the regulation of the neurite outgrowth and growth cone dynamics. In the present work, we have demonstrated that NO activates BACE1 translation in a pathway involving HRI kinase activation and eIF2α phosphorylation (Fig. 14).

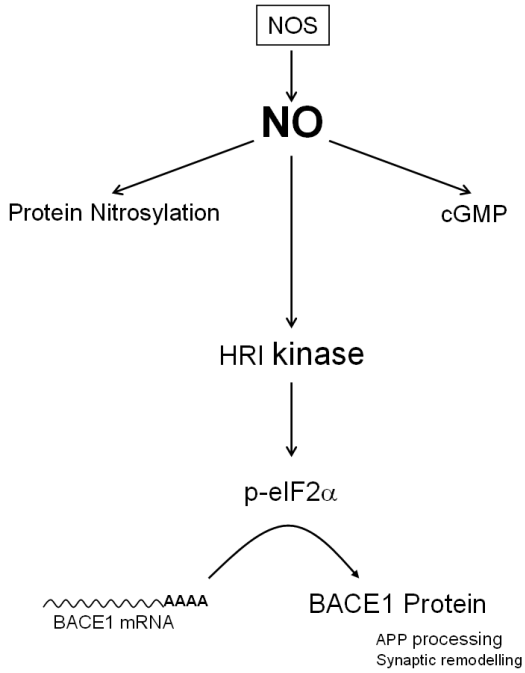


Figure 14. *NO/HRI/p-eIF2α signalling pathway*

The physiological significance NO/HRI/p-eIF2α pathway is supported by the individual role in learning and memory that has already been described individually for most of its components:

i) NO is a neurotransmitter with an important role in learning and memory (see chapter 2.1.6.)

ii) p-eIF2α (S51): The synaptic activity-dependent eIF2α phosphorylation at Serine 51 has been recently shown to be crucial in memory formation.

iii) BACE1: Initially discovered as the protease responsible for ß-secretase activity, its activity has been found to be necessary for memory and emotional related behavior and LTP.

HRI kinase has been mainly studied in the context of hemoglobin synthesis in reticulocytes although our data indicates that this kinase is also expressed in neuronal cells. Particularly, HRI is localized at synaptic density sites in hippocampal cells. HRI contains one or two heme group in its N-terminal domain [238,239] that behave as the receptor of NO. This enzyme structure has a direct analogy with guanylate cyclase, whose activation by NO remains the only recognized physiological NO signal transduction mechanism up to date. As HRI kinase, guanylate cyclase has a heme-binding domain that senses NO. The effector domain that in guanylate cyclase catalyzes cGMP production would correspond to the effector domain that in HRI kinase catalyses eIF2α phosphorylation.

The phosphorylation of eIF2α leads to an arrest of global protein synthesis. Nevertheless, neuron terminals have highly compartmentalized environments. Therefore, eIF2α phosphorylation occurs far away from the neuron soma without any consequence in the neuron somatic metabolism, and only altering temporary the genetic translational profile at synaptic sites, in an effect that can be easily delimited by the action of eIF2α phosphatases. Translational control is a crucial mechanism in highly polarized cells such as neurons, in which messenger RNAs are distributed asymmetrically at different subcellular locations. In neurons, new protein

synthesis is needed at active synaptic sites. Translational control of mRNAs located at synaptic sites allows rapid and specific genetic changes in a given synapse, in a process referred as "input specific synaptic remodelling". However, the nature of the intracellular signals that couple neurotransmitter and neurotrophins to the translational machinery is currently unknown. In that regard, we propose that NO can control translational initiation through direct activation of HRI kinase.

To the light of our results we hypothesize that NO signalling promote APP cleavage, inducing a transitory lowering in the adhesion force of the synaptic terminal necessary for structural rearrangements in the neuronal terminal.

The dysregulation of APP cleavage

Beyond the toxicity that Aß exerts on neuronal functions, we speculate that an overproduction of Aß peptide might be also mirroring a loss of function: the loss in the APP-mediated regulation of adhesion required for structural plasticity changes in neuron terminals.

With this work we propose that the GLU/NMDA/NO/HRI/p-eIF2α pathway activates the translation of BACE1 mRNA (and perhaps other downstream genes with analog 5'UTRs). Based on the involvement of the individual components of this cascade in learning and memory processes, we speculate that this pathway might play a role in synaptic plasticity through the control of gene translation at synaptic sites after neurotransmitter signalling. This signalling cascade uses eIF2α phosphorylation as a molecular switch that alternates its phosphorylated form with its non-phosphorylated one. The importance of eIF2α phosphorylation as a molecular switch in memory formation processes has already been demonstrated [240-243]. But it must be noticed that there is a

price to be paid for using eIF2α phosphorylation as a molecular switch for synaptic plasticity changes. This price implies that when the global neuronal stress increases over its baseline levels, the switch looses its physiological function. The eIF2α kinases (Fig. 10) are stress sensors that after being activated by any source of cellular stress phosphorylate eIF2α to arrest global protein synthesis. It is the biological cost of using a stress activated effector such as eIF2α for the fine regulation of translation initiation in the synapse.

Interestingly, some of the cellular stress factors that are commonly linked to AD induce eIF2α phosphorylation. It has two detrimental effects: i) Loss of control over BACE1 physiological activity, which is regulated by the synaptic activity-dependent phosphorylation of eIF2α. ii) Abnormal up-regulation of BACE1 translation, leading to Aß peptide over-production and AD pathology triggering.

HSV1 infection and AD

HSV1 was described to be a risk factor for AD [244]. However, it was not clear the relationship between HSV1 infection and the amyloidogenic cascade. We found that HSV1 infection in neuronal cells can activate an eIF2α kinase (PKR), leading to eIF2α phosphorylation and BACE1 translational activation (Fig. 15).

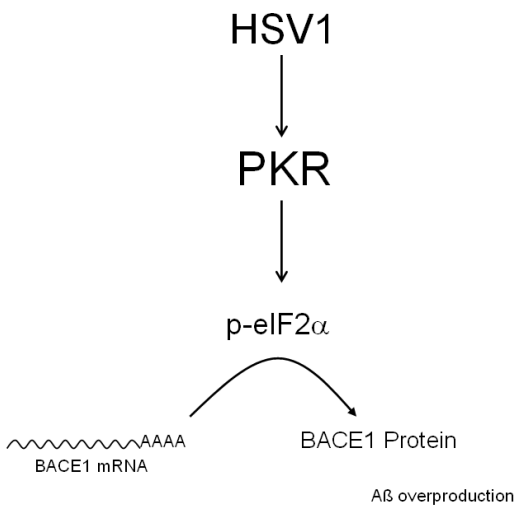


Figure 15. *HSV1 induces the phosphorylation of eIF2α, activating BACE1 translation*

These findings are reinforced by evidences of colocalization of HSV1 infected neurons with senile plaques in human brains [245,246]. Therefore, HSV1 reactivation in neuronal cells might be one of the stressful conditions that contribute to impair BACE1 tightly controlled translational activation and promoting, in addition, a triggering in Aß production. Unfortunately there is a lack of information on the mechanism that switch on neurotropic viruses during the life of an individual to asses whether HSV1 can remain active during sufficient time to produce a significant increase in Aß contributing to senile plaques formation.

Ca^{+2} dyshomeostasis, ER stress and AD

Accumulating evidence suggests that sustained disruption of Ca^{+2} homeostasis may play a role in AD pathogenesis. There are works reporting a direct role of Aß in the entry of calcium in neurons [247] although the formation of a pore by Aß is difficult to support as Aß is a

short peptide that cannot span through a biological membrane even when it forms oligomers. A direct action of Aß on a specific receptor or channel has not been demonstrated. The only reported effects of Aß in calcium homeostasis are based on its binding to NMDAR[248] or as a consequence of the oxidative stress in cells that indirectly increase the calcium intracellular load [249]. Following the observation that calcium release from the endoplasmic reticulum stores enhanced Aß production [250,251] it was proposed that the ß- and γ-secretase cleavages were modified by calcium-binding proteins or calcium-dependent proteases [252]. Interestingly, ER stress, missfolded proteins and Ca^{+2} dyshomeostasis activate one eIF2α kinase termed PERK (Fig. 16).

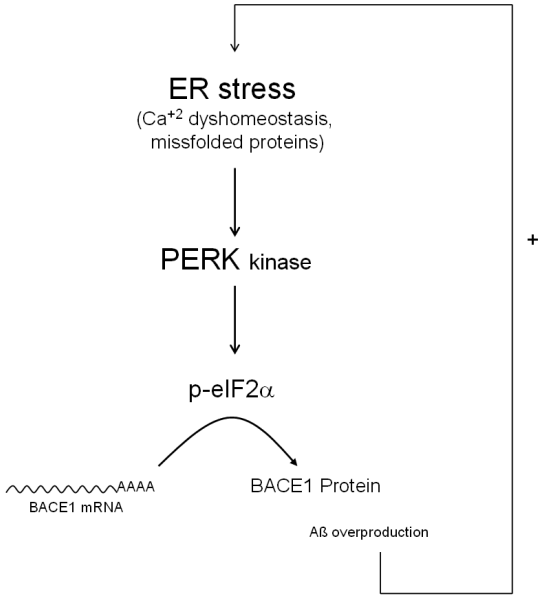


Figure 16. *eIF2α phosphorylation by PERK.* Interestingly, the Aß produced after BACE1 translation activation will further stimulate PERK activity, in an amplifying feed-back mechanism.

Therefore, BACE1 might be the APP-protease activated by Ca^{+2}, not directly through Ca^{+2}/calmodulin binding, but in an indirect way through the PERK mediated phosphorylation of eIF2α. In addition, some of the toxic effects attributed to Aß, as Ca^{+2} increase and ER stress, could trigger a feed-back effect towards an increased Aß production through PERK activation and eIF2α phosphorylation. Interestingly, PERK has been proposed as a direct Ca^{+2} dyshomeostasis sensor [253]. Furthermore, the unfolded protein response (UPR), among which PKR is one of the principal effector branch, is activated in AD [254] and the UPR machinery is localized in dendrites [255].

Brain ischemia and AD

There is an epidemiological link between Stroke and AD onset [173,174]. Studies based on different *in vivo* and *in vitro* brain ischemia models have reported an increase in BACE1 [175,176]. With similar approaches, PERK kinase has been found to be activated after brain ischemia and established as the responsible of the ischemia-induced translational arrest through eIF2α phosphorylation [177,256]. Putting together these evidences, we can consider the ischemic insult as another stress factor that by activating PERK contributes to the disruption of BACE1 translational regulation, promoting an up-regulation in BACE1 synthesis and triggering amyloidogenesis and AD onset as a consequence (Fig. 17).

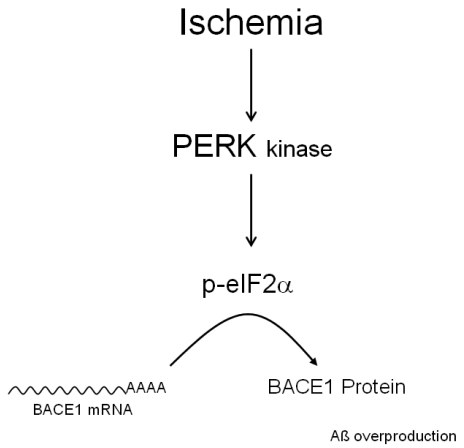


Figure 18. *The ischemic insult activates PERK kinase, leading to eIF2a phosphorylation and BACE1 translation activation.*

NO and Stroke

After ischemia the post-ischemic tissue responds increasing the NO production trying to recover the blood supply. It is a Janus mechanism since NO could act as a vasodilator but it also reacts with the free radicals that burst as a pathological consequence of the ischemia. The resulting peroxynitrite mediates nitrative damage in the proteins around the ischemic tissue. Even the plasmatic circulating proteins are affected by peroxynitrite which induces nytrotirosination. In our group we have previously studied the role of protein nitrotyrosination in AD, a neurodegenerative disease that shares with brain ischemia a central role for nitro-oxidative stress as a major harmful mechanism.

Here we focused in the study of fibrinogen nitrotyrosination since i) it has been reported that fibrinogen can be nitrotyrosinated in ischemic cardiac disease; ii) the only therapeutic approach in ischemic stroke is the

inhibition of clot formation in the early moments after the vascular accident.

We found that the impact of nitro-oxidative damage secondary to brain ischemia is not confined to the brain parenchyma, but it can spread to the circulating plasma proteins. Interestingly, nitrotyrosinated fibrinogen was detected as early as 3 h after the first stroke symptoms, suggesting a massive burst in peroxynitrite production after brain ischemia ─high enough to allow nitrotyrosinated proteins to reach significant levels in blood. New questions are opened by this finding, since it is hard to think that just a peripheral ischemic focus in brain is sufficient to produce the enormous amount of peroxynitrite presumably needed in order to detect nitrotyrosination in peripheral plasmatic proteins. It can be speculated that wide areas of the vascular system are involved of in the response to ischemia, based also in the limited pool of L-Arg, the substrate for NOS activity, that each cell has intracellularly [158]. Unfortunately we have not found data in the bibliography addressing this question.

We also found that the nitrotyrosination of fibrinogen changes its structure, something that will result in an impairment of its biological function. Fibrinogen nitrotyrosination makes fibrinogen more prone to form the clots [215]. But these clots made from nitrotyrosinated fibrinogen have a weak aggregative state that makes them easily deattachable from the vascular wall, increasing the risk of embolisms.

There are works reporting protection with antioxidants or peroxynitrite scavengers in stroke [257]. Consequently we have also described how the use of the antioxidant GSH can prevent fibrinogen nitrotyrosination *in vitro*. GSH is one of the most active antioxidants circulating in plasma. Interestingly, some polymorphisms in GSH metabolism associated proteins are associated to an increased risk of stroke [258,259]. With our work we contribute to the therapeutical bases of antioxidants in the treatment of

stroke attending to its beneficial role avoiding the nitrotyrosination of the fibrinogen.

Furthermore, we believe that nitrotyrosinated fibrinogen fulfills the criteria to be an early marker of ischemic stroke, favouring its rapid diagnosis. Indeed, a rapid and accurate diagnosis of ischemic stroke is needed in order to reduce mortality and avoid the substantial neurological deficits associated with late diagnosis.

Along the time, nitro-oxidative stress produced in stroke can trigger other pathological process. In fact, the risk of AD is increased after a stroke[173,174]. There are works reporting that brain ischemia induces the expression of BACE1[175] and the consequent Aß production. We speculate that a translational activation effect might be occurring over BACE1 mRNA through eIF2α phosphorylation. This is directly supported by our data showing that *in vitro* ischemia up-regulates BACE1 protein expression at short times (1h). However, the transactivation of BACE1 gene by HIF-1 after brain ischemia also contributes to enlarge the BACE1 mRNA pool [145].

Finally, even fibrinogen infiltration in the brain parenchyma could be contributing to AD onset as it has been previously proposed [260]. Consequently, a putative negative role for nitrotyrosinated fibrinogen in AD onset could be occurring.

Final consideration

The progress in Neurosciences has wide implications that overcome the circumscribed field of science or biomedical research. Indeed, the discoveries in this area of knowledge have a direct incidence in the whole modern conception of what is the human being.

The fact that neuroscientists study the relationship between mental processes (such as memory) and neurophysical processes has reopened a very old question addressing the interaction between *mind* and *matter*. This issue has been central in the History of phylosophy, stated in a variety of terms such as *soma-psique* or body-soul dilemmas. The epistemological answer to this question has been given mainly in two different and opposing perspectives. The *monism* posture argues that the essential nature of the mind is the same than that of matter, implying than the former can be reduced to the latter. According to monism, mind and matter are one same thing meaning that all mental processes can be explained with the laws that rule the behavior of the material world. Thomas Hobbes was a relevant phylosopher that defended the monistic posture with his *physical reductionism* understanding of the world. On the other hand, *dualism* is the philosophical posture that makes a distinction between the nature of matter and the nature of the mind, which is considered to overcome the limits of the physical world. Dualism has been defended by prime philosophers such as Socrates, Plato or Descartes.

The advances in modern neuroscience often lead to the monistic assumption that all mental processes are reducible to physico-chemical processes. But it is only an assumption. Obviously, a biological substrate is needed to support mental processes. An important part of this thesis has been centered precisely in the study of the biological substrates needed for memory formation. But the fact that mental processes require a biological substrate does not mean that they *are* the biological substrate itself. In my opinion, the current prevalent view supporting that mental processes are just the final *secretion* of a series of external stimuli processed by the brain machinery is only an assumption consequence of an argumentative slippery slope. Indeed, it is not a banal debate, as the possibility of human free will, and its crucial implications, entirely depends on the answer that is given to this issue.

5. Conclusions

1. Physiological concentrations of NO induce BACE1 translation in a pathway involving HRI kinase activation and eIF2α phosphorylation.

2. HRI kinase, eIF2α phosphorylated at Serine51 and BACE1 are localized at synaptic sites of hippocampal neurons.

3. The pathway glutamate-NO-HRI would be playing a relevant role in memory and learning processes due to its location and functional activity.

4. HSV1 can activate PKR despite the existence of host circumvention mechanisms directed to avoid PKR activation after a viral infection.

5. HSV1 infection activates BACE1 translation in a pathway involving PKR activation and eIF2α phosphorylation.

6. HSV1 infection induces an increase in Aß production which correlates with the presence of activated PKR in AD tissue.

7. Aß citotoxicity is mostly dependent on the production of free radicals due to its amyloid structure.

8. Antioxidants and other molecules that regulate antioxidant defences, such as neurotrophins, protect against Aß-induced damage.

9. Peroxynitrite strongly contributes to the Aß-induced cellular damages by inducing protein nitrotyrosination.

10. Brain ischemia induces nitro-oxidative stress that damages neurons, microglia and vascular cells.

11. Brain ischemia causes a significant nitrotyrosination of the circulating fibrinogen which alters its structure.

12. GSH prevents peroxynitrite-induced nitrotyrosination of fibrinogen *in vitro*.

6. References

1. Furchgott, R. F. & Zawadzki, J. V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376 (1980).

2. Ignarro, L. J. *et al.* Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* **218**, 739-749 (1981).

3. Palmer, R. M., Ferrige, A. G. & Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524-526 (1987).

4. Garthwaite, J., Charles, S. L. & Chess-Williams, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336**, 385-388 (1988).

5. Liu, X. *et al.* Nitric oxide diffusion rate is reduced in the aortic wall. *Biophys. J.* **94**, 1880-1889 (2008).

6. Ignarro, L. J. Endothelium-derived nitric oxide: actions and properties. *FASEB J.* **3**, 31-36 (1989).

7. Elfering, S. L., Sarkela, T. M. & Giulivi, C. Biochemistry of mitochondrial nitric-oxide synthase. *J. Biol. Chem.* **277**, 38079-38086 (2002).

8. Mungrue, I. N., Bredt, D. S., Stewart, D. J. & Husain, M. From molecules to mammals: what's NOS got to do with it? *Acta Physiol Scand.* **179**, 123-135 (2003).

9. Ebadi, M. & Sharma, S. K. Peroxynitrite and mitochondrial dysfunction in the pathogenesis of Parkinson's disease. *Antioxid. Redox. Signal.* **5**, 319-335 (2003).

10. Lowenstein, C. J., Glatt, C. S., Bredt, D. S. & Snyder, S. H. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. U. S. A* **89**, 6711-6715 (1992).

11. Mohammed, N. A. *et al.* Expression of nitric oxide synthase isoforms in human liver cirrhosis. *J. Pathol.* **200**, 647-655 (2003).

12. Kanno, K., Hirata, Y., Imai, T. & Marumo, F. Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension* **22**, 34-39 (1993).

13. Bucher, M. *et al.* Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteichoic acid. *FEBS Lett.* **412**, 511-514 (1997).

14. Xie, Q. W., Kashiwabara, Y. & Nathan, C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705-4708 (1994).

15. Kamijo, R. *et al.* Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* **263**, 1612-1615 (1994).

16. Hall, A. V. *et al.* Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J. Biol. Chem.* **269**, 33082-33090 (1994).

17. Bredt, D. S. & Snyder, S. H. Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* **13**, 301-313 (1994).

18. Cork, R. J. *et al.* A web-accessible digital atlas of the distribution of nitric oxide synthase in the mouse brain. *Prog. Brain Res.* **118**, 37-50 (1998).

19. Arbones, M. L. *et al.* Characteristics of nitric oxide synthase type I of rat cerebellar astrocytes. *Glia* **18**, 224-232 (1996).

20. Nozaki, K. *et al.* Possible origins and distribution of immunoreactive nitric oxide synthase-containing nerve fibers in cerebral arteries. *J. Cereb. Blood Flow Metab* **13**, 70-79 (1993).

21. Xu, K. Y., Huso, D. L., Dawson, T. M., Bredt, D. S. & Becker, L. C. Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A* **96**, 657-662 (1999).

22. Kobzik, L., Reid, M. B., Bredt, D. S. & Stamler, J. S. Nitric oxide in skeletal muscle. *Nature* **372**, 546-548 (1994).

23. Asano, K. *et al.* Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. U. S. A* **91**, 10089-10093 (1994).

24. Tojo, A. *et al.* Immunocytochemical localization of distinct isoforms of nitric oxide synthase in the juxtaglomerular apparatus of normal rat kidney. *J. Am. Soc. Nephrol.* **4**, 1438-1447 (1994).

25. Wang, Y., Goligorsky, M. S., Lin, M., Wilcox, J. N. & Marsden, P. A. A novel, testis-specific mRNA transcript encoding an NH2-terminal truncated nitric-oxide synthase. *J. Biol. Chem.* **272**, 11392-11401 (1997).

26. Magee, T. *et al.* Cloning of a novel neuronal nitric oxide synthase expressed in penis and lower urinary tract. *Biochem. Biophys. Res. Commun.* **226**, 145-151 (1996).

27. Shimizu, Y., Sakai, M., Umemura, Y. & Ueda, H. Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. *J. Dermatol.* **24**, 80-87 (1997).

28. Wang, Y., Newton, D. C. & Marsden, P. A. Neuronal NOS: gene structure, mRNA diversity, and functional relevance. *Crit Rev. Neurobiol.* **13**, 21-43 (1999).

29. Brenman, J. E. *et al.* Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* **84**, 757-767 (1996).

30. Brenman, J. E., Christopherson, K. S., Craven, S. E., McGee, A. W. & Bredt, D. S. Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. *J. Neurosci.* **16**, 7407-7415 (1996).

31. Kornau, H. C., Schenker, L. T., Kennedy, M. B. & Seeburg, P. H. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**, 1737-1740 (1995).

32. Zorumski, C. F. & Izumi, Y. Modulation of LTP induction by NMDA receptor activation and nitric oxide release. *Prog. Brain Res.* **118**, 173-182 (1998).

33. Hawkins, R. D., Son, H. & Arancio, O. Nitric oxide as a retrograde messenger during long-term potentiation in hippocampus. *Prog. Brain Res.* **118**, 155-172 (1998).

34. Marsden, P. A. *et al.* Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem.* **268**, 17478-17488 (1993).

35. Abe, K. *et al.* Upregulation of protein-tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. *Neurol. Res.* **19**, 124-128 (1997).

36. Colasanti, M. *et al.* Expression of a NOS-III-like protein in human astroglial cell culture. *Biochem. Biophys. Res. Commun.* **252**, 552-555 (1998).

37. Helfrich, M. H. *et al.* Expression of nitric oxide synthase isoforms in bone and bone cell cultures. *J. Bone Miner. Res.* **12**, 1108-1115 (1997).

38. Wang, R., Ghahary, A., Shen, Y. J., Scott, P. G. & Tredget, E. E. Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms. *J. Invest Dermatol.* **106**, 419-427 (1996).

39. Busconi, L. & Michel, T. Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization. *J. Biol. Chem.* **268**, 8410-8413 (1993).

40. Datta, S. R., Brunet, A. & Greenberg, M. E. Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905-2927 (1999).

41. Garcia-Cardena, G. *et al.* Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J. Biol. Chem.* **272**, 25437-25440 (1997).

42. Ignarro, L. J. Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* **41**, 485-490 (1991).

43. Butt, E., Geiger, J., Jarchau, T., Lohmann, S. M. & Walter, U. The cGMP-dependent protein kinase--gene, protein, and function. *Neurochem. Res.* **18**, 27-42 (1993).

44. Vaandrager, A. B. & de Jonge, H. R. Signalling by cGMP-dependent protein kinases. *Mol. Cell Biochem.* **157**, 23-30 (1996).

45. Ono, K. & Trautwein, W. Potentiation by cyclic GMP of beta-adrenergic effect on Ca2+ current in guinea-pig ventricular cells. *J. Physiol* **443**, 387-404 (1991).

46. Whalin, M. E., Scammell, J. G., Strada, S. J. & Thompson, W. J. Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells. *Mol. Pharmacol.* **39**, 711-717 (1991).

47. Stamler, J. S., Toone, E. J., Lipton, S. A. & Sucher, N. J. (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* **18**, 691-696 (1997).

48. Choi, Y. B. *et al.* Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat. Neurosci.* **3**, 15-21 (2000).

49. Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P. & Snyder, S. H. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat. Cell Biol.* **3**, 193-197 (2001).

50. Ravi, K., Brennan, L. A., Levic, S., Ross, P. A. & Black, S. M. S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc. Natl. Acad. Sci. U. S. A* **101**, 2619-2624 (2004).

51. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A* **87**, 1620-1624 (1990).

52. Huie, R. E. & Padmaja, S. The reaction of no with superoxide. *Free Radic. Res. Commun.* **18**, 195-199 (1993).

53. Cudd, A. & Fridovich, I. Electrostatic interactions in the reaction mechanism of bovine erythrocyte superoxide dismutase. *J. Biol. Chem.* **257**, 11443-11447 (1982).

54. Brennan, M. L. *et al.* A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* **277**, 17415-17427 (2002).

55. Souza, J. M., Daikhin, E., Yudkoff, M., Raman, C. S. & Ischiropoulos, H. Factors determining the selectivity of protein tyrosine nitration. *Arch. Biochem. Biophys.* **371**, 169-178 (1999).

56. Cassina, A. M. *et al.* Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* **275**, 21409-21415 (2000).

57. Amici, M., Lupidi, G., Angeletti, M., Fioretti, E. & Eleuteri, A. M. Peroxynitrite-induced oxidation and its effects on isolated proteasomal systems. *Free Radic. Biol. Med.* **34**, 987-996 (2003).

58. Newman, D. K. *et al.* Nitration of PECAM-1 ITIM tyrosines abrogates phosphorylation and SHP-2 binding. *Biochem. Biophys. Res. Commun.* **296**, 1171-1179 (2002).

59. Hink, U. *et al.* Role for peroxynitrite in the inhibition of prostacyclin synthase in nitrate tolerance. *J. Am. Coll. Cardiol.* **42**, 1826-1834 (2003).

60. Cassina, A. M. *et al.* Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* **275**, 21409-21415 (2000).

61. Ji, Y. & Bennett, B. M. Activation of microsomal glutathione s-transferase by peroxynitrite. *Mol. Pharmacol.* **63**, 136-146 (2003).

62. Zhang, J., Dawson, V. L., Dawson, T. M. & Snyder, S. H. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science* **263**, 687-689 (1994).

63. Guix, F. X. *et al.* Amyloid-dependent triosephosphate isomerase nitrotyrosination induces glycation and tau fibrillation. *Brain* **132**, 1335-1345 (2009).

64. Vadseth, C. *et al.* Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J. Biol. Chem.* **279**, 8820-8826 (2004).

65. Chabrier, P. E., merle-Pallardy, C. & Auguet, M. Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cell Mol. Life Sci.* **55**, 1029-1035 (1999).

66. Good, P. F., Werner, P., Hsu, A., Olanow, C. W. & Perl, D. P. Evidence of neuronal oxidative damage in Alzheimer's disease. *Am. J. Pathol.* **149**, 21-28 (1996).

67. Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S. & Perry, G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17**, 2653-2657 (1997).

68. Good, P. F., Hsu, A., Werner, P., Perl, D. P. & Olanow, C. W. Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **57**, 338-342 (1998).

69. Cookson, M. R. & Shaw, P. J. Oxidative stress and motor neurone disease. *Brain Pathol.* **9**, 165-186 (1999).

70. Park, J. H., Straub, V. A. & O'Shea, M. Anterograde signaling by nitric oxide: characterization and in vitro reconstitution of an identified nitrergic synapse. *J. Neurosci.* **18**, 5463-5476 (1998).

71. Nikonenko, I., Jourdain, P. & Muller, D. Presynaptic remodeling contributes to activity-dependent synaptogenesis. *J. Neurosci.* **23**, 8498-8505 (2003).

72. Susswein, A. J., Katzoff, A., Miller, N. & Hurwitz, I. Nitric oxide and memory. *Neuroscientist.* **10**, 153-162 (2004).

73. Arancio, O. *et al.* Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. *Cell* **87**, 1025-1035 (1996).

74. Son, H. *et al.* Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* **87**, 1015-1023 (1996).

75. Alzheimer, A. Über eine eigenartige Erkrankung der Hirnrinde. Allgemeine Z Psychiatrie Psychisch-Gerichtliche Med. 64, 146-148. 1907.

76. Glenner, G. G. & Wong, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885-890 (1984).

77. Masters, C. L. *et al.* Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. U. S. A* **82**, 4245-4249 (1985).

78. Kraeplin, E. Psychiatrie. Ein Lehrbuch für Studierende und Ärtze. Band.Klinische Psychiatrie. II. 1910. Leipzig, Verlag Johann Ambrosius Barth.

79. Alzheimer, A. Über eigenartige Krankheitsfalle des späteren Alters. Z die Gesamte Neurologie Psychiatrie 4, 385-456. 1911.

80. Vetrivel, K. S., Zhang, Y. W., Xu, H. & Thinakaran, G. Pathological and physiological functions of presenilins. *Mol. Neurodegener.* **1**, 4 (2006).

81. Sando, S. B. *et al.* APOE epsilon 4 lowers age at onset and is a high risk factor for Alzheimer's disease; a case control study from central Norway. *BMC. Neurol.* **8**, 9 (2008).

82. Bullido, M. J. *et al.* Double stranded RNA activated EIF2 alpha kinase (EIF2AK2; PKR) is associated with Alzheimer's disease. *Neurobiol. Aging* **29**, 1160-1166 (2008).

83. Hirtz, D. *et al.* How common are the "common" neurologic disorders? *Neurology* **68**, 326-337 (2007).

84. den Dunnen, W. F. *et al.* No disease in the brain of a 115-year-old woman. *Neurobiol. Aging* **29**, 1127-1132 (2008).

85. Kang, J. *et al.* The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733-736 (1987).

86. Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U. & Gajdusek, D. C. Characterization and chromosomal localization of a

cDNA encoding brain amyloid of Alzheimer's disease. *Science* **235**, 877-880 (1987).

87. Tanzi, R. E., Bird, E. D., Latt, S. A. & Neve, R. L. The amyloid beta protein gene is not duplicated in brains from patients with Alzheimer's disease. *Science* **238**, 666-669 (1987).

88. Robakis, N. K., Ramakrishna, N., Wolfe, G. & Wisniewski, H. M. Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proc. Natl. Acad. Sci. U. S. A* **84**, 4190-4194 (1987).

89. Olson, M. I. & Shaw, C. M. Presenile dementia and Alzheimer's disease in mongolism. *Brain* **92**, 147-156 (1969).

90. Goate, A. *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**, 704-706 (1991).

91. Mullan, M. *et al.* A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nat. Genet.* **1**, 345-347 (1992).

92. Hardy, J. Framing beta-amyloid. *Nat. Genet.* **1**, 233-234 (1992).

93. Scheuner, D. *et al.* Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2**, 864-870 (1996).

94. Borchelt, D. R. *et al.* Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. *Neuron* **17**, 1005-1013 (1996).

95. De Strooper, B. *et al.* Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-390 (1998).

96. De Strooper, B. *et al.* A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-522 (1999).

97. De Strooper, B. *et al.* Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-390 (1998).

98. Wisniewski, T., Ghiso, J. & Frangione, B. Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation. *Biochem. Biophys. Res. Commun.* **180**, 1528 (1991).

99. Wisniewski, T. & Frangione, B. Molecular biology of Alzheimer's amyloid--Dutch variant. *Mol. Neurobiol.* **6**, 75-86 (1992).

100. Nilsberth, C. *et al.* The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. *Nat. Neurosci.* **4**, 887-893 (2001).

101. Citron, M. *et al.* Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* **360**, 672-674 (1992).

102. Xu, X. Gamma-secretase catalyzes sequential cleavages of the AbetaPP transmembrane domain. *J. Alzheimers. Dis.* **16**, 211-224 (2009).

103. Suzuki, N. *et al.* An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* **264**, 1336-1340 (1994).

104. Jarrett, J. T., Berger, E. P. & Lansbury, P. T., Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693-4697 (1993).

105. Coulson, E. J., Paliga, K., Beyreuther, K. & Masters, C. L. What the evolution of the amyloid protein precursor supergene family tells us about its function. *Neurochem. Int.* **36**, 175-184 (2000).

106. Del Toro, D., Coma, M., Uribesalgo, I., Guix, F.X. & Muñoz, F.J. The amyloid beta-protein precursor and Alzheimer's disease. Therapeutic Approaches. Curr.Med.Chem.-Central Nervous System Agents 5, 271-283. 2005. Bentham Science Publishers Ltd. 2005.

107. Kang, J. & Muller-Hill, B. Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4(695) mRNA is predominantly produced in rat and human brain. *Biochem. Biophys. Res. Commun.* **166**, 1192-1200 (1990).

108. Wang, Y. & Ha, Y. The X-ray structure of an antiparallel dimer of the human amyloid precursor protein E2 domain. *Mol. Cell* **15**, 343-353 (2004).

109. Sisodia, S. S., Koo, E. H., Hoffman, P. N., Perry, G. & Price, D. L. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J. Neurosci.* **13**, 3136-3142 (1993).

110. De Strooper, B. & Annaert, W. Proteolytic processing and cell biological functions of the amyloid precursor protein. *J. Cell Sci.* **113 (Pt 11)**, 1857-1870 (2000).

111. Eggert, S. *et al.* The proteolytic processing of the amyloid precursor protein gene family members APLP-1 and APLP-2 involves alpha-, beta-, gamma-, and epsilon-like cleavages: modulation of APLP-1 processing by n-glycosylation. *J. Biol. Chem.* **279**, 18146-18156 (2004).

112. Raychaudhuri, M. & Mukhopadhyay, D. AICD and its adaptors - in search of new players. *J. Alzheimers. Dis.* **11**, 343-358 (2007).

113. Ling, Y., Morgan, K. & Kalsheker, N. Amyloid precursor protein (APP) and the biology of proteolytic processing: relevance to Alzheimer's disease. *Int. J. Biochem. Cell Biol.* **35**, 1505-1535 (2003).

114. Apelt, J., Bigl, M., Wunderlich, P. & Schliebs, R. Aging-related increase in oxidative stress correlates with developmental pattern of beta-secretase activity and beta-amyloid plaque formation in transgenic Tg2576 mice with Alzheimer-like pathology. *Int. J. Dev. Neurosci.* **22**, 475-484 (2004).

115. Simons, M. *et al.* Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *J. Neurosci.* **16**, 899-908 (1996).

116. Wang, J., Brunkan, A. L., Hecimovic, S., Walker, E. & Goate, A. Conserved "PAL" sequence in presenilins is essential for gamma-secretase activity, but not required for formation or stabilization of gamma-secretase complexes. *Neurobiol. Dis.* **15**, 654-666 (2004).

117. Allinson, T. M., Parkin, E. T., Turner, A. J. & Hooper, N. M. ADAMs family members as amyloid precursor protein alpha-secretases. *J. Neurosci. Res.* **74**, 342-352 (2003).

118. White, J. M. ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr. Opin. Cell Biol.* **15**, 598-606 (2003).

119. Karkkainen, I., Rybnikova, E., Pelto-Huikko, M. & Huovila, A. P. Metalloprotease-disintegrin (ADAM) genes are widely and differentially expressed in the adult CNS. *Mol. Cell Neurosci.* **15**, 547-560 (2000).

120. Postina, R. *et al.* A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. *J. Clin. Invest* **113**, 1456-1464 (2004).

121. Buxbaum, J. D. *et al.* Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of

the Alzheimer amyloid protein precursor. *J. Biol. Chem.* **273**, 27765-27767 (1998).

122. Lammich, S. *et al.* Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc. Natl. Acad. Sci. U. S. A* **96**, 3922-3927 (1999).

123. Scheuner, D. *et al.* Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2**, 864-870 (1996).

124. Kim, D. Y., Ingano, L. A. & Kovacs, D. M. Nectin-1alpha, an immunoglobulin-like receptor involved in the formation of synapses, is a substrate for presenilin/gamma-secretase-like cleavage. *J. Biol. Chem.* **277**, 49976-49981 (2002).

125. May, P., Reddy, Y. K. & Herz, J. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J. Biol. Chem.* **277**, 18736-18743 (2002).

126. Marambaud, P. *et al.* A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J.* **21**, 1948-1956 (2002).

127. Vassar, R. *et al.* Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735-741 (1999).

128. Farzan, M., Schnitzler, C. E., Vasilieva, N., Leung, D. & Choe, H. BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. *Proc. Natl. Acad. Sci. U. S. A* **97**, 9712-9717 (2000).

129. Sambamurti, K., Kinsey, R., Maloney, B., Ge, Y. W. & Lahiri, D. K. Gene structure and organization of the human beta-secretase (BACE) promoter. *FASEB J.* **18**, 1034-1036 (2004).

130. Christensen, M. A. *et al.* Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1. *Mol. Cell Biol.* **24**, 865-874 (2004).

131. O'Connor, T. *et al.* Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. *Neuron* **60**, 988-1009 (2008).

132. De Pietri, T. D. *et al.* Translational regulation of BACE-1 expression in neuronal and non-neuronal cells. *Nucleic Acids Res.* **32**, 1808-1817 (2004).

133. Lammich, S., Schobel, S., Zimmer, A. K., Lichtenthaler, S. F. & Haass, C. Expression of the Alzheimer protease BACE1 is suppressed via its 5'-untranslated region. *EMBO Rep.* **5**, 620-625 (2004).

134. Zhou, W. & Song, W. Leaky scanning and reinitiation regulate BACE1 gene expression. *Mol. Cell Biol.* **26**, 3353-3364 (2006).

135. Mihailovich, M., Thermann, R., Grohovaz, F., Hentze, M. W. & Zacchetti, D. Complex translational regulation of BACE1 involves upstream AUGs and stimulatory elements within the 5' untranslated region. *Nucleic Acids Res.* **35**, 2975-2985 (2007).

136. Willem, M., Lammich, S. & Haass, C. Function, regulation and therapeutic properties of beta-secretase (BACE1). *Semin. Cell Dev. Biol.* **20**, 175-182 (2009).

137. Willem, M. *et al.* Control of peripheral nerve myelination by the beta-secretase BACE1. *Science* **314**, 664-666 (2006).

138. Hu, X. *et al.* Bace1 modulates myelination in the central and peripheral nervous system. *Nat. Neurosci.* **9**, 1520-1525 (2006).

139. Laird, F. M. *et al.* BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J. Neurosci.* **25**, 11693-11709 (2005).

140. Wang, H., Song, L., Laird, F., Wong, P. C. & Lee, H. K. BACE1 knock-outs display deficits in activity-dependent potentiation of synaptic transmission at mossy fiber to CA3 synapses in the hippocampus. *J. Neurosci.* **28**, 8677-8681 (2008).

141. Ma, H. *et al.* Involvement of beta-site APP cleaving enzyme 1 (BACE1) in amyloid precursor protein-mediated enhancement of memory and activity-dependent synaptic plasticity. *Proc. Natl. Acad. Sci. U. S. A* **104**, 8167-8172 (2007).

142. Tamagno, E. *et al.* Oxidative stress increases expression and activity of BACE in NT2 neurons. *Neurobiol. Dis.* **10**, 279-288 (2002).

143. Coma, M. *et al.* Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells. *Neurobiol. Aging* **29**, 969-980 (2008).

144. Sun, X. *et al.* Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. *Proc. Natl. Acad. Sci. U. S. A* **103**, 18727-18732 (2006).

145. Zhang, X. *et al.* Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. *J. Biol. Chem.* **282**, 10873-10880 (2007).

146. Gatta, L. B., Albertini, A., Ravid, R. & Finazzi, D. Levels of beta-secretase BACE and alpha-secretase ADAM10 mRNAs in Alzheimer hippocampus. *Neuroreport* **13**, 2031-2033 (2002).

147. Preece, P. *et al.* Beta-secretase (BACE) and GSK-3 mRNA levels in Alzheimer's disease. *Brain Res. Mol. Brain Res.* **116**, 155-158 (2003).

148. Marcinkiewicz, M. & Seidah, N. G. Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. *J. Neurochem.* **75**, 2133-2143 (2000).

149. de Haro, C., Mendez, R. & Santoyo, J. The eIF-2alpha kinases and the control of protein synthesis. *FASEB J.* **10**, 1378-1387 (1996).

150. Hinnebusch, A. G. Translational control of GCN4: Gene-specific regulation by phosphorylation of eIF2. (Translational Control), 199-244. 1996. Coldspring Harbor Pres, J.W.B. Hershey, M.B. Mathews and N. Sonenberg, Editors.

151. Hinnebusch, A. G. Translational regulation of yeast GCN4. A window on factors that control initiator-trna binding to the ribosome. *J. Biol. Chem.* **272**, 21661-21664 (1997).

152. Smith, M. A. *et al.* Oxidative damage in Alzheimer's 24. *Nature* **382**, 120-121 (1996).

153. Lovell, M. A., Ehmann, W. D., Butler, S. M. & Markesbery, W. R. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease 2. *Neurology* **45**, 1594-1601 (1995).

154. Pike, C. J., Ramezan-Arab, N. & Cotman, C. W. Beta-amyloid neurotoxicity in vitro: evidence of oxidative stress but not protection by antioxidants. *J. Neurochem.* **69**, 1601-1611 (1997).

155. Huang, X. *et al.* The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction 34. *Biochemistry* **38**, 7609-7616 (1999).

156. Huang, X. *et al.* Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction
42. *J. Biol. Chem.* **274**, 37111-37116 (1999).

157. Ali, F. E. *et al.* Methionine regulates copper/hydrogen peroxide oxidation products of Abeta
41. *J. Pept. Sci.* **11**, 353-360 (2005).

158. Guix, F. X., Uribesalgo, I., Coma, M. & Munoz, F. J. The physiology and pathophysiology of nitric oxide in the brain. *Prog. Neurobiol.* **76**, 126-152 (2005).

159. Thorns, V., Hansen, L. & Masliah, E. nNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease
22. *Exp. Neurol.* **150**, 14-20 (1998).

160. Wallace, M. N., Geddes, J. G., Farquhar, D. A. & Masson, M. R. Nitric oxide synthase in reactive astrocytes adjacent to beta-amyloid plaques. *Exp. Neurol.* **144**, 266-272 (1997).

161. Luth, H. J., Holzer, M., Gartner, U., Staufenbiel, M. & Arendt, T. Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. *Brain Res.* **913**, 57-67 (2001).

162. Weldon, D. T. *et al.* Fibrillar beta-amyloid induces microglial phagocytosis, expression of inducible nitric oxide synthase, and loss of a select population of neurons in the rat CNS in vivo. *J. Neurosci.* **18**, 2161-2173 (1998).

163. Boje, K. M. & Arora, P. K. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res.* **587**, 250-256 (1992).

164. Akama, K. T., Albanese, C., Pestell, R. G. & Van Eldik, L. J. Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. *Proc. Natl. Acad. Sci. U. S. A* **95**, 5795-5800 (1998).

165. Hartlage-Rubsamen, M., Apelt, J. & Schliebs, R. Fibrillary beta-amyloid deposits are closely associated with atrophic nitric oxide synthase (NOS)-expressing neurons but do not upregulate the inducible NOS in transgenic Tg2576 mouse brain with Alzheimer pathology. *Neurosci. Lett.* **302**, 73-76 (2001).

166. Tran, M. H. *et al.* Tyrosine nitration of a synaptic protein synaptophysin contributes to amyloid beta-peptide-induced cholinergic dysfunction. *Mol. Psychiatry* **8**, 407-412 (2003).

167. Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S. & Perry, G. Widespread peroxynitrite-mediated damage in Alzheimer's disease
40. *J. Neurosci.* **17**, 2653-2657 (1997).

168. Hensley, K. *et al.* Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation
13. *J. Neurosci.* **18**, 8126-8132 (1998).

169. Tohgi, H. *et al.* Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease
1. *Neurosci. Lett.* **269**, 52-54 (1999).

170. Castegna, A. *et al.* Proteomic identification of nitrated proteins in Alzheimer's disease brain
43. *J. Neurochem.* **85**, 1394-1401 (2003).

171. Coma, M. *et al.* Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. *Brain* **128**, 1613-1621 (2005).

172. Masliah, E. *et al.* Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology* **56**, 127-129 (2001).

173. Tatemichi, T. K. *et al.* Dementia after stroke: baseline frequency, risks, and clinical features in a hospitalized cohort. *Neurology* **42**, 1185-1193 (1992).

174. Loeb, C., Gandolfo, C., Croce, R. & Conti, M. Dementia associated with lacunar infarction. *Stroke* **23**, 1225-1229 (1992).

175. Wen, Y., Onyewuchi, O., Yang, S., Liu, R. & Simpkins, J. W. Increased beta-secretase activity and expression in rats following transient cerebral ischemia. *Brain Res.* **1009**, 1-8 (2004).

176. Guglielmotto, M. *et al.* The up-regulation of BACE1 mediated by hypoxia and ischemic injury: role of oxidative stress and HIF1alpha. *J. Neurochem.* **108**, 1045-1056 (2009).

177. Owen, C. R. *et al.* PERK is responsible for the increased phosphorylation of eIF2alpha and the severe inhibition of protein synthesis after transient global brain ischemia. *J. Neurochem.* **94**, 1235-1242 (2005).

178. Pérez-Sempere, A. Ictus. (Chapter 1: Epidemiología del Ictus). 2000. Madrid, Ediciones Ergon.

179. Mostacero, E. A. & Tejero, C. Ictus. (Chapter 2: Fisiopatología y Bioquímica de la isquemia cerebral). 2000. Madrid, Ediciones Ergon.

180. Moro, M. A., Cardenas, A., Hurtado, O., Leza, J. C. & Lizasoain, I. Role of nitric oxide after brain ischaemia
36. *Cell Calcium* **36**, 265-275 (2004).

181. Ferrer, I. & Planas, A. M. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J. Neuropathol. Exp. Neurol.* **62**, 329-339 (2003).

182. Cuzzocrea, S., Riley, D. P., Caputi, A. P. & Salvemini, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol. Rev.* **53**, 135-159 (2001).

183. El Kossi, M. M. & Zakhary, M. M. Oxidative stress in the context of acute cerebrovascular stroke. *Stroke* **31**, 1889-1892 (2000).

184. Madamanchi, N. R., Vendrov, A. & Runge, M. S. Oxidative stress and vascular disease. *Arterioscler. Thromb. Vasc. Biol.* **25**, 29-38 (2005).

185. Chan, P. H. Role of oxidants in ischemic brain damage. *Stroke* **27**, 1124-1129 (1996).

186. Faraci, F. M. Oxidative stress: the curse that underlies cerebral vascular dysfunction? *Stroke* **36**, 186-188 (2005).

187. El Kossi, M. M. & Zakhary, M. M. Oxidative stress in the context of acute cerebrovascular stroke. *Stroke* **31**, 1889-1892 (2000).

188. Taffi, R. *et al.* Plasma levels of nitric oxide and stroke outcome. *J. Neurol.* **255**, 94-98 (2008).

189. Gao, L. & Mann, G. E. Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. *Cardiovasc. Res.* **82**, 9-20 (2009).

190. Harrison, D. G. & Gongora, M. C. Oxidative stress and hypertension. *Med. Clin. North Am.* **93**, 621-635 (2009).

191. Moro, M. A., Cardenas, A., Hurtado, O., Leza, J. C. & Lizasoain, I. Role of nitric oxide after brain ischaemia. *Cell Calcium* **36**, 265-275 (2004).

192. Choi, D. W. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623-634 (1988).

193. Meldrum, B. & Garthwaite, J. Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11**, 379-387 (1990).

194. Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H. & Dawson, T. M. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* **16**, 2479-2487 (1996).

195. Nowicki, J. P., Duval, D., Poignet, H. & Scatton, B. Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. *Eur. J. Pharmacol.* **204**, 339-340 (1991).

196. Buisson, A., Plotkine, M. & Boulu, R. G. The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia. *Br. J. Pharmacol.* **106**, 766-767 (1992).

197. Nagafuji, T., Matsui, T., Koide, T. & Asano, T. Blockade of nitric oxide formation by N omega-nitro-L-arginine mitigates ischemic brain edema and subsequent cerebral infarction in rats. *Neurosci. Lett.* **147**, 159-162 (1992).

198. Huang, Z. *et al.* Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265**, 1883-1885 (1994).

199. Garthwaite, J., Charles, S. L. & Chess-Williams, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336**, 385-388 (1988).

200. Huang, Z. *et al.* Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J. Cereb. Blood Flow Metab* **16**, 981-987 (1996).

201. Huang, Z. *et al.* Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265**, 1883-1885 (1994).

202. Goyagi, T. *et al.* Neuroprotective effect of sigma(1)-receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke* **32**, 1613-1620 (2001).

203. Ferriero, D. M., Holtzman, D. M., Black, S. M. & Sheldon, R. A. Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol. Dis.* **3**, 64-71 (1996).

204. Iadecola, C., Zhang, F., Xu, S., Casey, R. & Ross, M. E. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J. Cereb. Blood Flow Metab* **15**, 378-384 (1995).

205. De Alba, J. *et al.* Down-regulation of neuronal nitric oxide synthase by nitric oxide after oxygen-glucose deprivation in rat forebrain slices. *J. Neurochem.* **72**, 248-254 (1999).

206. Grandati, M. *et al.* Calcium-independent NO-synthase activity and nitrites/nitrates production in transient focal cerebral ischaemia in mice. *Br. J. Pharmacol.* **122**, 625-630 (1997).

207. Cardenas, A. *et al.* Protective effect of N-(3-(aminomethyl)benzyl) acetamidine, an inducible nitric oxide synthase inhibitor, in brain slices exposed to oxygen-glucose deprivation. *Eur. J. Pharmacol.* **354**, 161-165 (1998).

208. Iadecola, C., Zhang, F., Casey, R., Nagayama, M. & Ross, M. E. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J. Neurosci.* **17**, 9157-9164 (1997).

209. Eliasson, M. J. *et al.* Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J. Neurosci.* **19**, 5910-5918 (1999).

210. Huang, Z. *et al.* Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine
42. *J. Cereb. Blood Flow Metab* **16**, 981-987 (1996).

211. Robert-Ebadi, H., de Moerloose. P., El Khorassani M., El Khattab M. & Neerman-Arbez, M. A novel frameshift mutation in FGA accounting for congenital afibrinogenemia predicted to encode an aberrant peptide terminating 158 amino acids downstream. *Blood Coagul. Fibrinolysis* **20**, 385-387 (2009).

212. Hanss, M. & Biot, F. A database for human fibrinogen variants. *Ann. N. Y. Acad. Sci.* **936**, 89-90 (2001).

213. Acharya, S. S. & Dimichele, D. M. Rare inherited disorders of fibrinogen. *Haemophilia.* **14**, 1151-1158 (2008).

214. Doolittle, R. F., Hong, S. & Wilcox, D. Evolution of the fibrinogen gamma' chain: implications for the binding of factor XIII, thrombin and platelets. *J. Thromb. Haemost.* **7**, 1431-1433 (2009).

215. Vadseth, C. *et al.* Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species 17. *J. Biol. Chem.* **279**, 8820-8826 (2004).

216. Huang, Z. *et al.* Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265**, 1883-1885 (1994).

217. Goyagi, T. *et al.* Neuroprotective effect of sigma(1)-receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke* **32**, 1613-1620 (2001).

218. Ferriero, D. M., Holtzman, D. M., Black, S. M. & Sheldon, R. A. Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol. Dis.* **3**, 64-71 (1996).

219. Lambert, M. P. *et al.* Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. U. S. A* **95**, 6448-6453 (1998).

220. Hartley, D. M. *et al.* Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* **19**, 8876-8884 (1999).

221. Hsia, A. Y. *et al.* Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. U. S. A* **96**, 3228-3233 (1999).

222. Mucke, L. *et al.* High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050-4058 (2000).

223. Lewis, J. *et al.* Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**, 1487-1491 (2001).

224. Hardy, J. & Selkoe, D. J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-356 (2002).

225. Lambert, M. P. *et al.* Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. U. S. A* **95**, 6448-6453 (1998).

226. Walsh, D. M. *et al.* Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**, 535-539 (2002).

227. Nordberg, A., Rinne, J. O., Kadir, A. & Langstrom, B. The use of PET in Alzheimer disease. *Nat. Rev. Neurol.* **6**, 78-87 (2010).

228. Tanzi, R. E. *et al.* The genetic defect in familial Alzheimer's disease is not tightly linked to the amyloid beta-protein gene. *Nature* **329**, 156-157 (1987).

229. Wasco, W. *et al.* Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor. *Nat. Genet.* **5**, 95-100 (1993).

230. Herms, J. *et al.* Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. *EMBO J.* **23**, 4106-4115 (2004).

231. Ferreira, A., Caceres, A. & Kosik, K. S. Intraneuronal compartments of the amyloid precursor protein. *J. Neurosci.* **13**, 3112-3123 (1993).

232. Small, D. H. *et al.* A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J. Neurosci.* **14**, 2117-2127 (1994).

233. Sabo, S. L., Ikin, A. F., Buxbaum, J. D. & Greengard, P. The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses in vitro and in vivo. *J. Neurosci.* **23**, 5407-5415 (2003).

234. Priller, C. *et al.* Synapse formation and function is modulated by the amyloid precursor protein. *J. Neurosci.* **26**, 7212-7221 (2006).

235. Young-Pearse, T. L. *et al.* A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J. Neurosci.* **27**, 14459-14469 (2007).

236. Dawson, G. R. *et al.* Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience* **90**, 1-13 (1999).

237. Cirrito, J. R. *et al.* Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo. *Neuron* **48**, 913-922 (2005).

238. Uma, S., Matts, R. L., Guo, Y., White, S. & Chen, J. J. The N-terminal region of the heme-regulated eIF2alpha kinase is an autonomous heme binding domain. *Eur. J. Biochem.* **267**, 498-506 (2000).

239. Miksanova, M. *et al.* Characterization of heme-regulated eIF2alpha kinase: roles of the N-terminal domain in the oligomeric state, heme binding, catalysis, and inhibition. *Biochemistry* **45**, 9894-9905 (2006).

240. Costa-Mattioli, M. *et al.* Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature* **436**, 1166-1173 (2005).

241. Costa-Mattioli, M. & Sonenberg, N. Translational control of long-term synaptic plasticity and memory storage by eIF2alpha. *Crit Rev. Neurobiol.* **18**, 187-195 (2006).

242. Costa-Mattioli, M. *et al.* eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. *Cell* **129**, 195-206 (2007).

243. Costa-Mattioli, M., Sossin, W. S., Klann, E. & Sonenberg, N. Translational control of long-lasting synaptic plasticity and memory. *Neuron* **61**, 10-26 (2009).

244. Itzhaki, R. F., Lin, W. R., Wilcock, G. K. & Faragher, B. HSV-1 and risk of Alzheimer's disease. *Lancet* **352**, 238 (1998).

245. Jamieson, G. A., Maitland, N. J., Wilcock, G. K., Craske, J. & Itzhaki, R. F. Latent herpes simplex virus type 1 in normal and Alzheimer's disease brains. *J. Med. Virol.* **33**, 224-227 (1991).

246. Wozniak, M. A., Mee, A. P. & Itzhaki, R. F. Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* **217**, 131-138 (2009).

247. Jang, H., Zheng, J. & Nussinov, R. Models of beta-amyloid ion channels in the membrane suggest that channel formation in the bilayer is a dynamic process. *Biophys. J.* **93**, 1938-1949 (2007).

248. Shankar, G. M. *et al.* Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J. Neurosci.* **27**, 2866-2875 (2007).

249. De Felice, F. G. *et al.* Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J. Biol. Chem.* **282**, 11590-11601 (2007).

250. Querfurth, H. W., Jiang, J., Geiger, J. D. & Selkoe, D. J. Caffeine stimulates amyloid beta-peptide release from beta-amyloid precursor protein-transfected HEK293 cells. *J. Neurochem.* **69**, 1580-1591 (1997).

251. Cheung, K. H. *et al.* Mechanism of Ca2+ disruption in Alzheimer's disease by presenilin regulation of InsP3 receptor channel gating. *Neuron* **58**, 871-883 (2008).

252. Querfurth, H. W. & Selkoe, D. J. Calcium ionophore increases amyloid beta peptide production by cultured cells. *Biochemistry* **33**, 4550-4561 (1994).

253. Liang, S. H., Zhang, W., McGrath, B. C., Zhang, P. & Cavener, D. R. PERK (eIF2alpha kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homoeostasis. *Biochem. J.* **393**, 201-209 (2006).

254. Hoozemans, J. J. *et al.* The unfolded protein response is activated in Alzheimer's disease. *Acta Neuropathol.* **110**, 165-172 (2005).

255. Murakami, T., Hino, S. I., Saito, A. & Imaizumi, K. Endoplasmic reticulum stress response in dendrites of cultured primary neurons. *Neuroscience* **146**, 1-8 (2007).

256. Kumar, R. *et al.* Brain ischemia and reperfusion activates the eukaryotic initiation factor 2alpha kinase, PERK. *J. Neurochem.* **77**, 1418-1421 (2001).

257. Cuzzocrea, S., Riley, D. P., Caputi, A. P. & Salvemini, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury
10. *Pharmacol. Rev.* **53**, 135-159 (2001).

258. Kolsch, H. *et al.* Polymorphisms in glutathione S-transferase omega-1 and AD, vascular dementia, and stroke. *Neurology* **63**, 2255-2260 (2004).

259. Voetsch, B. *et al.* Promoter polymorphisms in the plasma glutathione peroxidase (GPx-3) gene: a novel risk factor for arterial ischemic stroke among young adults and children. *Stroke* **38**, 41-49 (2007).

260. Cortes-Canteli, M. & Strickland, S. Fibrinogen, a possible key player in Alzheimer's disease. *J. Thromb. Haemost.* **7 Suppl 1**, 146-150 (2009).