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PRESENILIN-2 AND CALCIUM HANDLING IN FAMILIAL ALZHEIMER'S DISEASE: A GENETICALLY ENCODED Ca^{2+} PROBES-BASED STUDY ROLE OF PS2 STRUCTURE, ER Ca^{2+}-LEAK PATHWAYS AND ER-MITOCHONDRIA INTERPLAY

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ABBREVIATIONS

Aβ: Amyloid β peptide

AD: Alzheimer's Disease

Aeq: Aequorin

AICD: APP IntraCellular Domain

Aph-1: Antherior Pharynx-defective 1

APP: Amyloid Precursor Protein

BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

Bk: Bradikinin

Ca^{2+}: calcium

$[Ca^{2+}]$: Ca^{2+} concentration

$[Ca^{2+}]_{cyt}$: cytosolic Ca^{2+} concentration

$[Ca^{2+}]_{ER}$: ER Ca^{2+} concentration

$[Ca^{2+}]_{ext}$: extracellular Ca^{2+} concentration

$[Ca^{2+}]_{Golgi}$: Golgi apparatus Ca^{2+} concentration

$[Ca^{2+}]_{mit}$: mitochondrial Ca^{2+} concentration

CaM: Calmodulin

CCE: Capacitative Ca^{2+} Entry

CFP: Cyan Fluorescent Protein

CICR: Ca^{2+}-Induced-Ca^{2+}-Release

CPA: cyclopiazonic acid

CRT: Calreticulin

CTF: C-terminal fragment

DAG: diacylglicerol

EGTA: glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid

ER: Endoplasmic Reticulum

FAD: Familial Alzheimer's Disease

FL: full length

FRET: Fluorescence Resonance Energy Transfer

GFP: Green Fluorescent Protein

GPCR: G-protein-coupled Receptor

HEDTA: 2-[2-[bis(carboxymethyl)amino]ethyl-(2-hydroxyethyl)amino]acetic acid

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HMW: high molecular weight

IMM: inner mitochondrial membrane

IMS: intermembrane space

IP_3: inositol-1,4,5-triphosphate

IP_3R: IP_3 Receptor

MAM: Mitochondria-Associate-Membrane

MEF: mouse embryonic fibroblast

Mfn: Mitofusin

mKRB: modified Krebs–Ringer buffer

MW: molecular weight

NCT: Nicastrin

NCX: Na^+/Ca^{2+} exchanger

NICD: Notch IntraCellular Domain

NTF: N-terminal fragment

OMM: outer mitochondrial membrane

Pen-2: Presenilin Enhancer-2

PHF: Paired Helical Filament

PIP_2: phosphatidylinositol-4,5-biphosphate

PLCβ: Phospholipase-Cβ

PM: Plasma Membrane

PMCA: Plasma-Membrane Ca^{2+}-ATPase

PS1: Presenilin-1

PS2: Presenilin-2

RFP: Red Fluorescent Protein

ROC: Receptor-Operated Channel

RTC: Ribosomal-Translocon Complex

RTK: Receptor-Tyrosin-Kinase

RyR: Ryanodine Receptor

SERCA: Sarco/Endoplasmatic Ca^{2+}-ATPase

siRNA: small interfering RNA

SMOC: Second-Messenger-Operated Channel

SOC: Store-Operated Channel

STIM: STromal Interaction Molecule

TMD: trans-membrane domain

TRIS : 1,1,1-tris(hydroxymethyl)-methanamine

VDAC: Voltage Dependent Anion Channel

VOC: Voltage-Operated Channel

wt: wild type

YFP: Yellow Fluorescent Protein

SUMMARY

Calcium (Ca^{2+}) is a key second messenger in living cells and it regulates a multitude of cell functions; this means as well that a dysregulation in its signaling cascade can be detrimental for cell fate. Ca^{2+} mishandling has been proposed as a causative mechanism for most neurodegenerative diseases and in particular for Alzheimer's Disease (AD).

From the middle '80s, alterations in Ca^{2+} dynamics were noticed in fibroblasts from AD patients, but extensive studies on AD and Ca^{2+} homeostasis started only after the identification of mutations linked to Familial Alzheimer's Disease (FAD) in three genes, *app*, *psen-1* and *psen-2*, coding for Amyloid Precursors Protein (APP), Presenilin-1 and Presenilin-2 (PS1, PS2).

Mutations in these genes caused alterations in the cleavage of APP by a PS1- or PS2-containing enzyme, thus leading to an increase in the ratio between the two main peptides finally derived from APP maturation, called Aβ40 and Aβ42, in favor of the latter, the most toxic and most prone to aggregation specie; this in turn would increase the deposition of "Amyloid Plaques", one of the principal histopathological feature of AD. Up to now, the generation of Aβ42 peptides, its oligomers and finally amyloid plaques is the core of the most widely accepted pathogenic hypothesis for AD, the "Amyloid Cascade Hypothesis".

Considering Ca^{2+} homeostasis, most of the attention has been paid to the effect of PS1 (and only lately of PS2) mutations. Initially, most works reported an increase in the Ca^{2+} released in the cytosolic compartment from ER upon stimulation in cells expressing FAD-PS(1), thus suggesting an increase in ER Ca^{2+} loading, the "Ca^{2+} Overload Hypothesis". Although supported by several groups for many years, this hypothesis has never been undisputedly accepted, since some data were clearly in contrast with it, especially those considering PS2 mutations.

Recently a strong evidence in support of "Ca^{2+} overload hypothesis" came from works suggesting that wt PSs can form low conductance Ca^{2+} channels in the ER membrane, providing most of the constitutive Ca^{2+} leak from the organelle; this function would be compromised by FAD mutations, that would thus lead to an ER Ca^{2+} overload. Again, these data have not been collectively accepted and other groups provided alternative explanations for the enhanced Ca^{2+} release in FAD-PSs (mostly PS1) expressing cells, such as enhanced

Ryanodine Receptor (RyR) activation, augmented IP_3 Receptor (IP_3R) opening probability or potentiated SERCA activity. Interestingly, some of these data were no more suggesting an increased ER Ca^{2+} content (and so what is properly named "Ca^{2+} overload") but rather an exaggerated Ca^{2+} release from the store, with unchanged (or even reduced) ER Ca^{2+} concentration.

The effect of PS2 mutations has been less studied and is more controversial. Fibroblasts from patients bearing PS2 mutations showed reductions in Ca^{2+} release upon stimulation and actually data obtained by employing ER-targeted Ca^{2+} probes (e.g. ER-targeted aequorin) in FAD-PS2 stable clones, as well as cell lines transiently-transfected with different FAD-PS2 mutations, showed a reduction in ER Ca^{2+} concentration.

Starting from these results, demonstrating that the expression of PS2 bearing FAD-linked mutations dampens the intracellular Ca^{2+} stores, the molecular mechanisms behind this phenomenon have been taken under investigation.

In particular, three different aspects of PS2 effect on Ca^{2+} handling have been analyzed, mostly employing organelle-targeted aequorin Ca^{2+} probes: (i) the role of PS2 conformation on its effect in reducing the ER Ca^{2+} level; PSs are in fact synthesized as holoprotein but soon after their incorporation into γ-secretase complex undergo a maturation cleavage to their active, dimeric form; ii) the involvement of the Ca^{2+} leak pathway across the ER membrane in PS2's effect and more specifically of three different possible leak pathways proposed by literature, RyR, IP_3R and the Ribosomal-Translocon complex (RTC); (iii) the role of PS2 in regulating the interplay between ER and mitochondria, a critical feature in cell life.

Experiments on cells devoid of endogenous PS1 and PS2 and transfected with different PS2 constructs (resulting in the expression of the full length PS2, the dimeric PS2 or both) demonstrated that the full length conformation of this protein is necessary for the reduction in ER Ca^{2+} level linked to its expression; moreover, it has been shown that increasing, by different approaches, the endogenous level of the full length PS2 decreases ER Ca^{2+} content.

Data aimed at measuring the decay rate of ER Ca^{2+} in control cells compared with FAD-PS2 expressing cells revealed an increased Ca^{2+} leak in the latter, and so the possible involvement of RyR, IP_3R and RTC was investigated both by pharmacological (application of RyR inhibitor Dantrolene, IP_3R antagonist Heparin, RTC opener/closer Puromycin/Anisomycin) or genetic (siRNA against IP_3R-1 and -3 isoforms) approaches,

showing that the PS2-induced ER Ca^{2+} reduction is at least partially mediated by RyR and IP_3R, but not by RTC.

Mitochondrial uptake of Ca^{2+} released from ER was evaluated. When mitochondrial Ca^{2+} peaks were measured in PS2-expressing and control cells in a condition in which their cytosolic peaks were comparable (by partially pre-emptying control cells), an increase in mitochondrial Ca^{2+} uptake was observed for cells over-expressing PS2 wt and, more prominently, FAD-PS2. This was not due to a direct effect of PS2 on mitochondrial uptake machinery but to an increased interaction between these organelles and the ER, as demonstrated by evaluating the two organelles proximity by confocal microscopy. By down-regulating PS2 with siRNA, it was also shown that endogenous PS2 can control ER-mitochondria interaction. These results were confirmed by employing FRET-based "Cameleon" cytosolic and mitochondrial Ca^{2+} probes on single-cells experiments.

Altogether these findings provide new insights into the PS2 effect on Ca^{2+} homeostasis in Familial Alzheimer's Disease and, more specifically, on the role of PS2 conformation and on the involvement of RyR and IP_3R in its effect. A new aspect of the PS2 control of cell (and in particular Ca^{2+}) dynamics is also emerging since it is here shown, for the first time, that this protein can influence the interplay between ER and mitochondria and that FAD-mutations affect this interaction, opening new directions for the investigation of the effects of PSs' mutations in Familial Alzheimer's Disease.

RIASSUNTO

Il calcio (Ca^{2+}) è un secondo messaggero chiave nelle cellule e regola una moltitudine di funzioni cellulari; questo significa che una dis-regolazione nella sua cascata di trasduzione del segnale può essere deleteria per il destino della cellula. Difetti nella regolazione del Ca^{2+} sono stati proposti come possibili cause della maggior parte delle malattie neurodegenerative e in particolare della Malattia di Alzheimer (AD).

Sin dalla metà degli anni ’80 furono notate alterazioni nelle dimamiche intracellulari del Ca^{2+} in fibroblasti ottenuti da pazienti affetti da AD, ma studi sistematici sulla relazione tra AD e omeostasi del Ca^{2+} cominciarono solo dopo l’identificazione di mutazioni legate alle forme familiari di AD (FAD) a carico di tre geni, *app*, *psen-1* e *psen-2*, codificanti per la Proteina Precursone dell’Amiloide (APP), Presenilina-1 e Presenilina-2 (PS1, PS2).

Mutazioni in questi geni causano alterazioni nel taglio di APP ad opera di un enzima contenente PS1 o PS2, le quali comportano quindi un aumento nel rapporto tra i due principali peptidi derivanti dalla maturazione di APP, chiamati Aβ40 e Aβ42, in favore di quest’ultimo, la specie più tossica e con maggiore tendenza all’aggregazione; questo d’altra parte aumenta la deposizioni delle “Placche Amiloidi”, una delle caratteristiche istopatologiche dell’AD. Attualmente, la generazione dei peptidi Aβ42, dei suoi oligomeri e infine delle placche amiloidi è alla base della principale ipostesi sulla patogenesi dell’AD, l’“*Amyloid Cascade Hypothesis*”.

Riguardo l’omeostasi del Ca^{2+}, la maggior parte dell’attenzione è stata rivolta all’effetto delle mutazioni in PS1 (e solo successivamente in PS2). Inizialmente la maggior parte dei lavori riportava un incremento degli aumenti citosolici di Ca^{2+} indotti dalla stimolazione del rilascio dal reticolo endoplasmatico (ER) in cellule esprimenti mutazioni in PS1 associate a FAD (FAD-PS1), e perciò suggerivano un aumento nel contenuto di Ca^{2+} del ER, “*Ca^{2+} Overload Hypothesis*”. Nonostante sia stata sostenuta da molti gruppi per diversi anni questa ipotesi non è mai stata indiscutibilmente accettata, poiché esistevano alcuni dati evidentemente in contrasto con essa, soprattutto considerando mutazioni a carico di PS2.

Recentemente l’ipotesi del “*Ca^{2+} Overload*” ha ricevuto forte supporto dalla dimostrazione che le PS nella loro forma *wild type* (wt) formano canali a bassa conduttanza per il Ca^{2+} nella membrana del ER, rappresentando la maggior parte della via di fuga (*leak*)

costitutiva del Ca^{2+} dallo stesso organulo; questa funzione sarebbe compromessa dalle mutazioni associate a FAD, che quindi porterebbero ad un sovraccarico di Ca^{2+} nel ER. Anche in questo caso, questi dati non sono stati universalmente accettati e altri gruppi hanno fornito spiegazioni alternative all'aumentato rilascio di Ca^{2+} nelle cellule esprimenti mutazioni associate a FAD a carico delle PS (soprattutto PS1), cioè un'aumentata attivazione del Recettore Rianodinico (RyR), una maggiore probabilità di apertura del Recettore dell'IP_3 (IP_3R) o un potenziamento dell'attività della Ca^{2+}-ATPasi del ER (SERCA). È interessante osservate che alcuni di questi dati non riportano più un aumento nel contenuto di Ca^{2+} del ER (cioè quello che è propriamente definito "*Ca^{2+} Overload*") ma piuttosto un rilascio esagerato di Ca^{2+} dallo stesso deposito, con una concentrazione al suo interno inalterata o persino diminuita.

L'effetto delle mutazioni in PS2 è stato meno studiato ed è più dibattuto. Fibroblasti ottenuti da pazienti affetti da mutazioni FAD in PS2 hanno rivelato un ridotto rilascio di Ca^{2+} dal ER in seguito a stimolazione, e dati ottenuti utilizzando sonde in grado di misurare direttamente la concentrazione di Ca^{2+} nel ER (in particolare basate su equorina modificata indirizzata al ER) hanno dimostrato una diminuzione nella concentrazione di Ca^{2+} nel ER sia in cloni stabili che in linee cellulari esprimenti mutazioni FAD in PS2.

A partire da questi risultati, i quali dimostrano che l'espressione di PS2 recanti mutazioni associate a FAD riduce il contenuto di Ca^{2+} dei depositi intracellulari, si è deciso di investigare i meccanismi molecolari alla base di questo fenomeno.

In particolare, sono stati investigati, soprattutto mediante l'impiego di equorine indirizzate a diversi compartimenti cellulari, tre diversi aspetti dell'effetto di PS2 sull'omeostasi del Ca^{2+}: i) il ruolo della conformazione di PS2 nel suo effetto di riduzione del livello di Ca^{2+} del ER; le PS sono infatti sintetizzate come proteine intere ma subiscono rapidamente una maturazione mediante taglio ad una forma dimerica quando sono incorporate nel complesso γ-secretasico; ii) il coinvolgimento del *leak* di Ca^{2+} attraverso la membrane del ER nell'effetto di PS2, e più in particolare di tre diverse possibili "vie di fuga" suggerite in letteratura, RyR, IP_3R e il complesso Ribosoma-Traslocone (RTC); iii) il ruolo di PS2 nell'interazione tra ER e mitocondri, un aspetto centrale nella fisiologia della cellula.

Esperimenti su cellule prive di PS1 e PS2 endogene transfettate con diversi costrutti di PS2 (in grado di dare l'espressione di PS2 intera, dimerica o di entrambe) hanno dimostrato che la sua conformazione intera è necessaria per la riduzione del contenuto di Ca^{2+} del ER

indotta da PS2; inoltre, è stato dimostrato che un aumento nel livello endogeno di PS2 intera diminuisce il livello di Ca^{2+} nel ER.

Dati mirati a misurare la velocità di uscita del Ca^{2+} attraverso la membrana del ER hanno evidenziato un *leak* di ioni Ca^{2+} aumentato in cellule esprimenti PS2 associate a FAD rispetto ai controlli, e quindi si è investigato il possibile coinvolgimento di RyR, IP_3R e RTC sia con approcci di tipo farmacologico (impiego dell'inibitore di RyR Dantrolene, dell'antagosista di IP_3 Eparina, di Puromicina e Anisomicina, due agenti che bloccano RTC in conformazione aperta o chiusa rispettivamente) che di tipo genetico (siRNA contro le isoforme 1 e 3 di IP_3R al fine di diminuirne il livello proteico), arrivando a dimostrare che la riduzione del contenuto di Ca^{2+} del ER indotta da PS2 è almeno parzialmente mediata da RyR e IP_3R ma non da RTC.

È stato inoltre valutato l'accumulo da parte dei mitocondri del Ca^{2+} rilasciato dal ER. Quando sono stati misurati i picchi di Ca^{2+} mitocondriale in cellule esprimenti PS2 e cellule di controllo in condizioni in cui il rilascio citosolico era confrontabile (le cellule di controllo venivano parzialmente pre-svuotate) si è osservato un aumento nella captazione di Ca^{2+} da parte dei mitocondri in cellule esprimenti PS2 wt e, in modo più pronunciato, PS2 associata a FAD. Questo fenomeno non è dovuto a un effetto diretto di PS2 sul meccanismo di ingresso di Ca^{2+} nei mitocondri, bensì ad un aumento nell'interazione tra i mitocondri stessi e il ER, come è stato dimostrato misurando la prossimità dei due organuli mediante microscopia confocale. L'abbattimento dei livelli proteici di PS2 grazie a siRNA ha inoltre evidenziato che la PS2 endogena può controllare il grado di interazione tra ER e mitocondri. Questi risultati sono stati infine confermati da esperimenti fatti con sonde "Cameleon" per il Ca^{2+}, basate sul fenomeno del FRET.

Complessivamente questi dati forniscono nuovi elementi sull'effetto di PS2 sull'omeostasi del Ca^{2+} nelle forme familiari della Patologia di Alzheimer e, più in dettaglio, sul ruolo della conformazione di PS2 e sul coinvolgimento di RyR e IP_3R nel suo effeto. Un nuovo aspetto del controllo da parte di PS2 sulle dinamiche cellulari (e del Ca^{2+} in particolare) viene inoltre riportato per la prima volta, cioè che questa proteina può regolare la relazione tra ER e mitocondri, e che mutazioni FAD a suo carico influenzano questa interazione; questo suggerisce ovviamente nuove possibili vie di indagine sull'effetto delle mutazioni a carico delle PS nelle forme familiari di AD.

INTRODUCTION

The name “Presenilin” appeared for the first time in the scientific background in August 1995, on Nature (Rogaev et al., 1995), following the identification of a gene on chromosome 1, whose mutations were associated with genetic forms of Alzheimer’s Disease (AD), the third gene linked to familial cases of this pathology identified in a few years.

The quest for genetic mutations linked to AD had its first hit in 1991, when Goate and her colleagues found a mutation in the gene coding for Amyloid Precursor Protein (APP) on chromosome 21 in two families with hereditary, early-onset, forms of AD (Goate et al., 1991); however, since the late 80s it was reported a linkage between a genetic defects on such chromosome and familial AD (St George-Hyslop et al., 1987).

The second hit came in June 1995 (Sherrington et al., 1995), as a gene bearing missense AD-linked mutations, named “S182”, was identified in chromosome 14, as already suggested by Schellenberg and colleagues (Schellenberg et al., 1992). Two months later the third gene mentioned above (initially named “E5-1”, Rogaev et al., 1995) was discovered because of its homology with “S182”, and two AD-linked mutations in its sequence were found. Clearly, the name “presenilins” was due to the early-onset of the genetic forms of AD, and in October of the same year the two genes, S182 and E5-1, were yet distinctly named “Presenilin-1” and “Presenilin-2” by the “Alzheimer’s Disease Collaborative Group” (Nat Genet., 1995). By the end of 1995, a tentative protein structural conformation was proposed, suggesting at least seven trans-membrane domains (TMD) and an hydrophilic loop between TMD6 and 7 (HL6-7) and revealing two regions more affected by mutations (TMD2 and HL6-7). Afterwards, more than 160 missense AD-linked mutations were reported (more than 150 for Preseninilin-1 and 10 for Presenilin-2, Ertekin-Taner, 2007); the hypothesis about the function(s) of the two proteins were however less than speculative (Van Broeckhoven, 1995).

Since then, Presenilins have been revealing themselves as complex and many-faced proteins and are still (and even more) object of investigations and controversies.

Although Presenilins’ discovery was mainly due to their involvement in the pathogenesis of AD, which will be discussed in more detail hereafter, growing number of evidence enlightened their multifunctional, and still not fully understood, role in several cell physiological and pathological processes, as will be reviewed in the following sections.

Calcium signalling is one of these Presenilin-modulated process, and actually the effect of Presenilin-2 on calcium homeostasis is the topic of this work, summarizing the results obtained during three years of research in Prof. Pozzan's Lab under the supervision of Dr. Paola Pizzo and Dr. Cristina Fasolato.

Alzheimer's Disease

Alzheimer's Disease (AD) is the most common adult neurodegenerative disease, counting more than 20 million cases worldwide, with a massive social and economic impact.

The clinical features of Alzheimer's Disease pathology can be described following the progression of the pathology: at the pre-dementia stage patients show very mild cognitive impairment in acquiring new information and other demanding cognitive tasks (e.g. planning complex actions) can be compromised; in the mild-dementia stage significant deficits in learning and memory are the most prominent clinical features; communication can begin to suffer from shrinking vocabulary, decreasing word fluency and less precise language; also spatial disorientation usually starts at this stage. In the moderate-dementia stage a severe impairment in recent memory makes patients appear to "live in the past", difficulties in language get more obvious as well as in writing and reading; spatial disorientation increases, while inability to recognize familiar faces, illusionary misidentifications and hallucinations begin; aimless and restless activity is common, as well as aggressive behaviours; finally, in the severe-dementia stage even early biographical memories are lost, language is reduced to single words and patients completely lost their self-sufficiency (Förstl & Kurz, 1999).

AD prevalence steeply increases in people over 80 years, and indeed age is closely linked to the pathology, together with environmental and health risk-factors such as socio-economic conditions, diet, environmental enrichment, obesity, diabetes or hyperlipidemia. The age of onset is also a relevant parameter that allows to distinguish between "late-onset" AD (onset over 60 years) and "early onset" AD (onset before 60 years), that counts for the 6-7 % of the total AD cases. AD cases are mostly sporadic, but in a small percentage (presumably less than 1 %, indeed the 13 % of "early onset" cases) are dominantly inherited: as described above, the three genes responsible for the Familial Alzheimer's Disease (FAD) were identified in the early 90s: the 81 % of FAD cases are due to mutations in PS1 gene (with the most aggressive

phenotype and the earliest onset), the 14 % to mutations in APP gene and only the 6 % to mutations in PS2 gene (Ertekin-Taner, 2007; Geodert & Spillantini, 2006).

Histopathological features of AD: neurofibrillary tangles and amyloid plaques

The first case of AD was reported in 1907 by Alois Alzheimer (Alzheimer, 1907), who described its defining neuropathological features: neurofibrillary tangles and amyloid (also called neuritic) plaques (Fig. 1). At variance with other neurodegenerative diseases, the neuronal loss due to Alzheimer's Disease is extensive and diffuse, with a clear cortical atrophy, and not only one neuronal population is affected, and this can explain its heterogeneity. Noteworthy, clinical manifestations and hystopathological features of sporadic and familial AD are the same, albeit some FAD cases can occasionally present also some distinctive clinical signs, such as myoclonus or seizure (Ertekin-Taner, 2007; Selkoe, 2001).

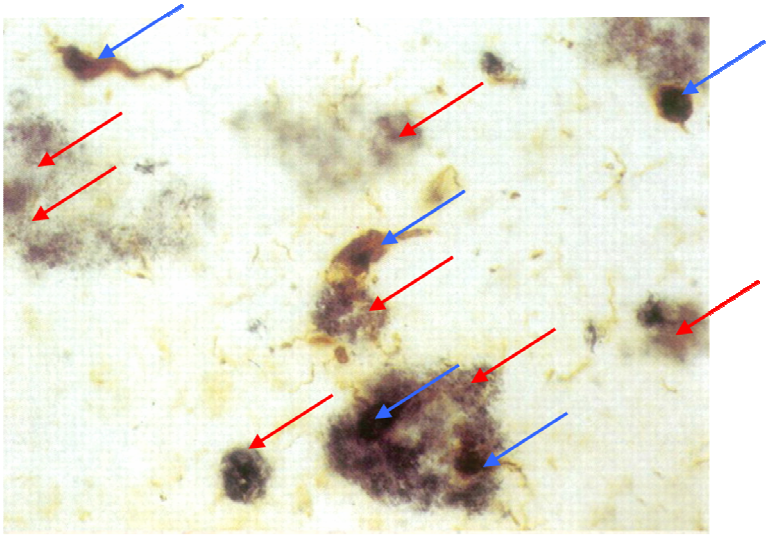

Fig. 1: AD histopathological features: neurofibrillary tangles (blue arrows) and amyloid plaques (red arrows, modified form Goedert & Spillantini, 2006).

Neurofibrillay tangles are large bundles of abnormal filaments with paired helical morphology (paired helical filaments – PHFs) composed of hyper-phosphorylated tau protein (Goedert & Spillantini, 2006; Selkoe, 2001), a microtubule associated protein involved in microtubules dynamics and axonal transport, whose functions are tightly regulated by phosphorylation (Johnson & Stoothoff, 2004).

Amyloid Plaques are microscopic (cross-sectional area usually between 10 and 120 μm) *loci* of extracellular deposition of amyloid-β petide (Aβ), principally in filamentous form, usually associated with axonal and dendritic injuries (dilated and tortuous neuronal processes, with ultrastructural abnormalities like enlarged lysosomes, numerous mitochondria and paired helical filaments); also non-neuronal cells are involved in plaque formation: microglia cells are often found adjacent to the central amyloid core, while astrocytes can form a ring outside of the plaque (Selkoe, 2001). This lesions probably evolve gradually and slowly during months or years and it is thought that their formation starts with "diffuse plaques" (immature lesions), becoming then "primitive plaques" and finally amyloid plaques, both characterized by the presence of Aβ fibrils, dystrophic neuritis and glial reaction. It should be noticed however that Aβ is a normal secreted product in brain during ageing and is not a unique feature of AD-affected brains, unless an abnormal accumulation occurs (Morishima-Kawashima & Ihara, 2002).

Aβ peptides derive from two sequential cleavages of their precursor protein, the already mentioned Amyloid Precurson Protein (APP). APP is a type I integral membrane protein, with a large extracellular N-terminal domain and a short cytosolic C-terminal domain (Kang et al., 1987; Dyrks et al. 1988); although the transcript of the gene can be alternatively spliced, the major isoform in neurons is the one composed by 695 aminoacid (aa), also called APP695. This protein has been suggested to have several biological functions, such as cell adhesion, neuronal survival and migration, neurite outgrowth, axonal transport, synaptogenesis and synaptic plasticity (reviewed in Jacobsen & Iverfeldt, 2009; Thinakaran & Koo, 2008); recently, a first physiological ligand for APP involved in cell adhesion has been reported (TAG-1, Ma et al., 2008).

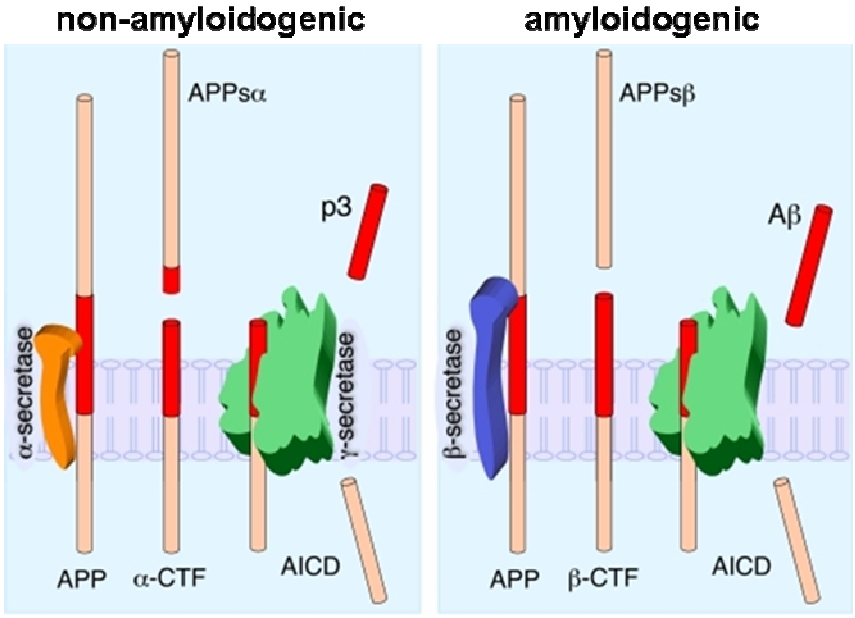

Fig. 2: APP processing by α- or β-secretase and γ-secretase (non-amyloidogenic and amyloidogenic pathway, respectively), see text for details (modified from Thinakaran & Koo, 2008)

APP is initially cleaved by two alternative enzymes, α- or β-secretase, respectively identified as a member of the zinc-metalloproteinases (e.g. ADAM17, 9 or 10, see Allinson et al., 2003) and the aspartyl protease BACE (Vassar, 2004), whose cleavage sites are both on the extracellular domain, in proximity of the unique transmembrane domain (TMD). Both the enzymes release two soluble fragments in the extracellular space, ("ectodomain shedding"), named sAPPα or sAPPβ, and generate two alternative membrane anchored peptides, respectively C83 and C99, which are subsequently substrates of the second cleavage of APP, by the action of γ-secretase (Fig. 2, Thinakaran & Koo, 2008). Moreover, two additional cleavage sites on APP, called ε and ζ, have been identified and attributed to γ-secretase (Weidemann et al., 2002; Zhao et al., 2004).

γ-secretase

γ-secretase proteolyses the two membrane-bound C-99 and C-83 peptides within their TMD: the cytosolic product of the cleavage is the same, a 6 kDa peptide called AICD (APP IntraCellular Domain) which has been reported to regulate gene transcription (von Rotz et al., 2004; Cao & Südhof, 2001; Kimberly et al., 2001), while the fragments released on the other

side of the membrane are different: from C83 (and so upon α-secretase cleavage) a 3 kDa peptide (p3) is generated; from C99 (generated by β-secreatse cleavage) the noxious Aβ peptide is produced. It should be noticed that, since α- and β-secretases compete for APP, the two different cleavage pathways are mutually exclusive and are distinctly called amyloidogenic (β-cleavage followed by γ-cleavage) and non-amyloidogenic pathway (α-cleavage followed by γ-cleavage) (Thinakaran & Koo, 2008).

The ability of γ-secretase to perform an intramembrane protein cleavage is shared with few other enzymes and collectively they are defined as "intramembrane-cleaving proteases" (I-CLiPs), mediating the Regulated Intramembrane Proteolysis (RIP): site 2 proteases (S2P) and rhomboids are both serine proteases, while signal peptide peptidases (SPP) and γ-secretase are membrane embedded aspartyl proteases. All I-CLiPs hydrolyse within their substrates' TMD and the protease active site is located within the intramembrane region or at the membrane-cytosol interface of the enzyme (McCarthy et al., 2009; Dries & Yu, 2008).

γ-secretase is generally considered a promiscuous enzyme for two reasons: first, it can cleave a variety of different substrates, ranging from the here discussed APP to Notch receptor, from the cell-adhesion molecules N- and E-cadherins, to p75 neurotrophin co-receptor (extensively reviewed in McCarthy et al., 2009), showing very few common structural features, like being type I transmembrane proteins and requiring ectodomain shedding (Hemming et al., 2008); indeed, such a loose specificity suggests that it could also act as a "membrane proteasome", facilitating membrane-protein turnover (Kopan & Ilagan, 2004). Second, γ-secretase does not show a unique cleavage site on APP, generating thus Aβ peptides with various length (Wang et al., 1996) with the two more prominent species being Aβ40 (40 aa long) and Aβ42 (42 aa long) that account respectively for the 90% and the 5-10% of total Aβ peptides in normal brains (Fig. 3); as it will be discussed later, a common feature of FAD mutations in PSs and APP genes is an increase in the ratio between Aβ42 and Aβ40 (Aβ42/Aβ40 ratio) (Walsh & Selkoe, 2007; Bentahir et al., 2006; Scheuner et al., 1996).

Apart from APP, Notch is probably the most extensively studied substrate of γ-secretase. Notch is a ligand-activated transmembrane receptor with an essential role in development and in brain function (Costa et al., 2005; Lai, 2004) whose signalling pathway requires the release in the cytosol, and the following translocation to the nucleus, of its intracellular domain

(NICD), produced upon the activation of a protease (Schroeter et al., 1998, Struhl & Adachi, 1998) that was soon identified as γ-secretase (De Strooper et al., 1999).

The molecular identity of γ-secretase is still not completely clear, but in the last few years four proteins emerged as the minimal, necessary and sufficient components for γ-secretase activity: Presenilin (either PS1 or PS2), Nicastrin (NCT), Antherior Pharynx-defective 1 (Aph-1) and Presenilin Enhancer-2 (Pen-2) (Fraering et al., 2004; Edbauer et al., 2003); these four proteins can be respectively identified as "the Substrate Receptor" (NCT), "the Stabilizer" (Aph-1), "the Linchpin" (Pen-2) and lastly PS, when endoproteolysed to form a dimeric, mature protein (see below), is the Active Site (Fig. 3, Dries & Yu, 2008). The precise stoichiometry of these components in the complex was not undoubtedly described, until recently it has been demonstrated that "active γ-secretase complexes contain only one of each component"(Spasic & Annaert, 2008; Sato et al., 2007).

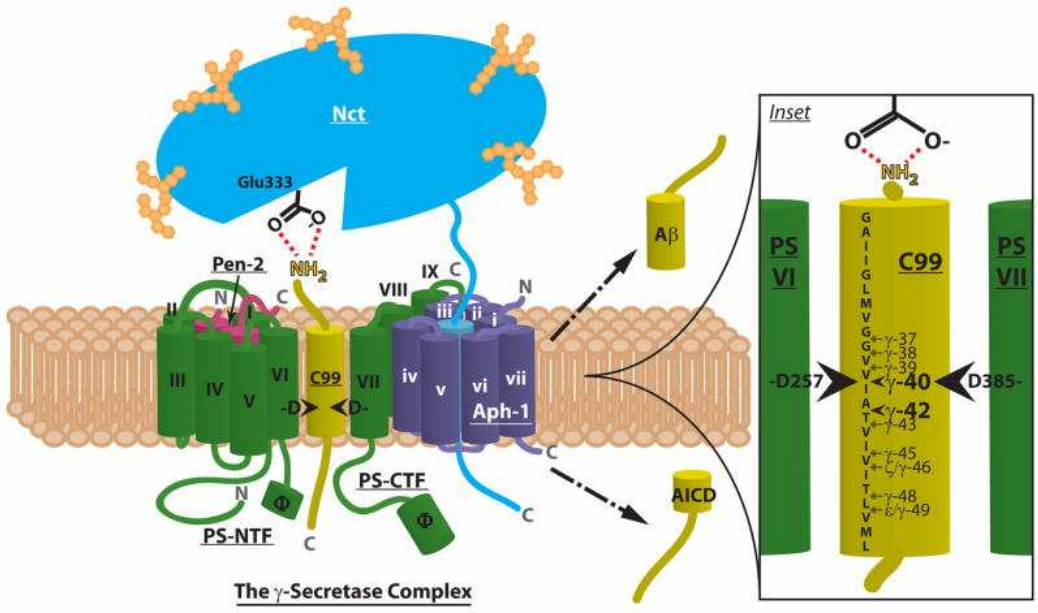

Fig. 3: Composition of the active γ-secretase complex and sites of γ-secretase cleavage in APP (Dries & Yu, 2008).

Presenilins

Presenilin-1 and Presenilin-2 are respectively 467 and 448 aminoacid long (MW around 50 kDa) membrane proteins with ten hydrophobic domains, according to their sequence. The topology of these domains has been extensively studied and explained with several models

proposing a structure with 6 to 9 TMDs; until recent years the 8 TMDs model was the most popular (Dewji, 2005; Brunkan & Goate, 2005 Li & Greenwald, 1998; Doan et al., 1996) but currently a 9 TMDs structure, with N-terminus oriented towards the cytosol, the C-terminus towards to the extracellular space and the tenth hydrophobic domain in a cytosolic loop between TMD6 and TMD7 is widely accepted (Fig. 4; Spasic et al., 2006; Laudon et al. 2005).

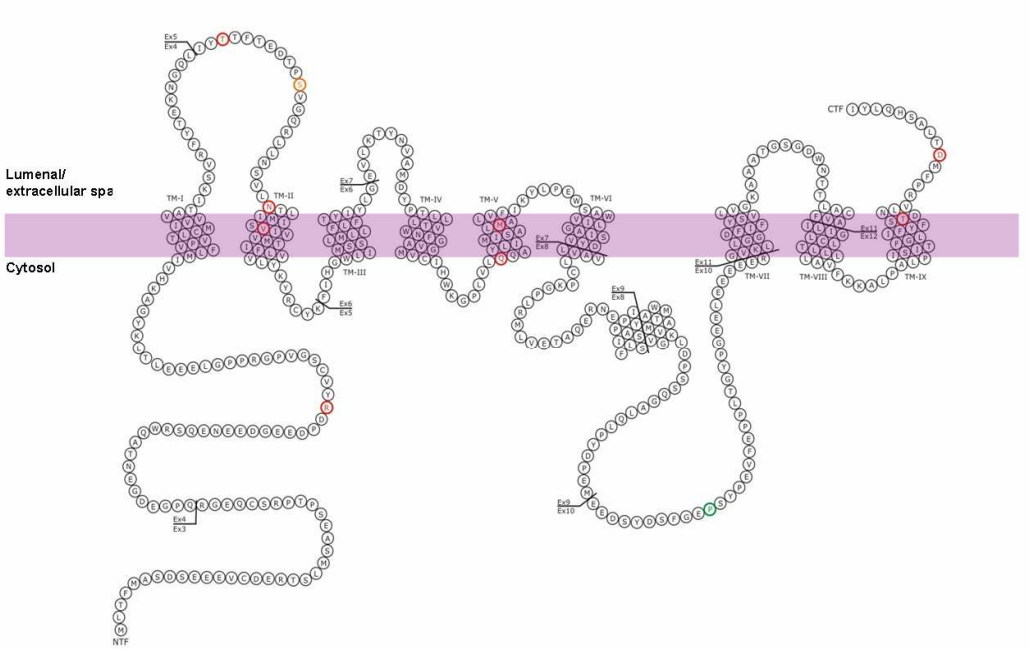

Fig. 4: 9 TMDs structure of Presenilins; the tenth hydrophobic domain is located in a large cytosolic loop (modified from www.molgen.ua.ac.be/ADMutations).

As stated above, Presenilins are the active site of γ-secretase complex. A first evidence of their implication in Notch signalling came from studies showing developmental deficits in PS1 KO mice (Wong et al., 1997; Shen et al., 1997) and that Notch undergoes a cleavage similar to the γ-secretase-mediated cleavage of APP, depending on PS1 (De Strooper et al., 1999). Consequently, it became clear that Presenilins were necessary also for APP cleavage and more generally for γ-secretase activity, identifying the two aspartates on TMD6 and TMD7 (respectively D257 and D385 in PS1) as critical residues for the proteolysis (Kimberly et al., 2000; Herreman et al., 2000; Wolfe et al., 1999; De Strooper et al., 1998). It became also evident that the enzyme responsible for the final cleavage of Notch and APP was actually

the same, γ-secretase, depending on Presenilins activity (Berezovska et al., 2000 and 2001; Zhang et al., 2000).

Presenilin is firstly synthetized as a holoprotein but rapidly undergoes endoproteolysis at the level of the cytosolic loop between TMD6 and TMD7, generating a 30 kDa N-terminal fragment and a 20 kDa C-terminal fragment (NTF and CTF respectively) (Thinakaran et al., 1996); the levels of PS fragments are tightly regulated and not all the full length (FL) PS is cleaved because of competition for limiting factors (Thinakaran et al., 1997); the unprocessed holoprotein is rapidly degraded while in the γ-secretase complex the active form of the protein is formed by the stable heterodimer of PS NTF and CTF, what is considered the "mature" form of PS (Levitan et al., 2001; Steiner et al., 1998). The identity of the enzyme known as "Presenilinase" is not completely clear, but multiple evidence points at PS itself as the probable candidate, thus describing the maturation cleavage as an auto-endoproteolysis and actually catalitically inactive PS mutants do not undergo proper maturation (Brunkan et al. 2005a; Kimberly et al., 2000). However, well characterized γ-secretase inhibitors show different potency, pharmacologically distinct, in blocking γ-secretase or presenilinase activity (Campbell et al., 2003 and 2002). Probably PS exists in two different conformations: "presenilinase" conformation precedes the maturation process and perform auto-endoproteolysis; after that, PS switches to mature "γ-secretase conformation" and can cleave other substrates (Brunkan et al., 2005b).

Presenilin's partners in γ-secretase

Although Presenilins were identified as the protease responsible for APP (and Notch) peptide bond hydrolysis within the lipid bilayer, it appeared quite soon evident that they function in High Molecular Weight (HMW) complexes and that they require interactions with other proteins to act properly (Yu et al., 1998).

In the year 2000 co-immunoprecipitation (with PS1) assay and mass spectrometry allowed the identification of a novel type I transmembrane protein named "Nicastrin" (NCT), 709 aa long, with a large N-terminal extracellular glycosylated domain; Nicastrin was shown to interact with both PS1 and PS2 and to modulate γ-secretase cleavage of APP (Yu et al., 2000).

Since its discovery, it was clear that NCT couldn't be a mere substrate of Presenilins and it was alternatively supposed to have a role in binding the substrates of γ-secretase or in

regulating the activity of the complex. It was 5 years later that Shah and colleagues (Shah et al., 2005) elegantly demonstrated that Nicastrin acts as the γ-secretase-substrate receptor through its N-terminal domain. In particular, through its large DAP domain, containing the conserved DYIGS motif, it recognizes the ectodomain-shedding generated amino-terminal stubs of type I transmembrane proteins and recruits them into the enzymatic complex.

However, it soon became clear that additional proteins should act as limiting factors for the complete maturation of over-expressed Presenilins and Nicastrin (Arawaka et al., 2002). Genetic screening in *C. elegans* allowed the identification of two proteins, the above mentioned Aph-1 and Pen-2 (Francis et al., 2002; Goutte et al., 2002), as well as their human (Aph1a/b), murine (Aph1a/b/c) and drosophila homologues. The seven-transmembrane-spanning protein Aph-1 has a molecular weight of 30 kDa and is the most stable component of the γ-secretase complex, probably stabilizing PS holoprotein and serving as initial scaffold for the assembly of the complex; on the other hand, the small (101 aa long) hairpin two-membrane-spanning Pen-2 is necessary for PS endoproteolysis and enhances γ-secretase activity by optimizing the active site conformation (Takasugi et al. 2003, but see also Dries & Yu, 2008 and Tolia & De Strooper, 2009). Recently, two conserved histidines in Aph-1 TM5 and TM6 domains (His-171 and His-197) have been shown to be relevant for PS catalytic activity (Pardossi-Piquard et al., 2009). Two different studies, on the other side, provided evidence that an "NF" motif in PS(1) TMD4 is required for interaction with Pen-2 and that TMD1 of Pen-2 is critical for PS endoproteolysis (Kim & Sisodia 2005 a,b)

Albeit these four proteins seem to be the essential for the γ-secretase molecular machinery, it is plausible that other proteins can bind and modulate the activity of the complex; the basic requirements for additional components are: direct interactions with at least one of the four proved components, presence in HMW complexes and direct modulation of secretase activity. Several proteins have been proposed as possible modulators, e.g. CD147, phospholipase D, calsenilin, and members of X11/mint family (Dries & Yu, 2008; Verdile et al., 2006); between these, two seem particularly interesting: transmembrane trafficking protein 21 (Tmp21), a type I transmembrane protein of the p24 cargo-protein family involved in quality control and protein transport at the level of ER and Golgi apparatus, has been reported to immunoprecipitate with all the four γ-secretase components and to influence secretase function (Chen et al., 2006); also Rer1p (retrieval to ER 1 protein) is involved in quality control and seems to control the proper assembly of γ-secretase and its trafficking

from the ER (Spasic et al., 2007; Kaether et al., 2007). Another protein reported to bind Presenilins, to influence their maturation and to modulate γ-secretase activity is Ubiquilin (1 or 2, Massey et al., 2005).

γ-secretase is usually referred as a unique enzyme, but actually this is not totally correct: probably several (at least six) γ-secretase complexes exist, formed by Presenilin-1 or Presenilin-2, Aph1a/b, and their splicing variants, and the other components (Shirotani et al., 2007 and 2004). The functional relevance of this heterogeneity has however still to be disclosed.

γ-secretase structure, assembly and trafficking

The crystal structure of the multi-TMDs γ-secretase is still lacking, but in the last years some progresses have been made about its 3D structure. It should be noticed that the peptide bond hydrolysis operated by the enzyme requires the access of water molecules to the catalytic site and this could be explained by the existence of a water-containing cavity inside the enzyme, as multiple evidence recently stated (Tolia et al. 2008; Sato et al., 2006); moreover, electron microscopy revealed that at the top and at the bottom of this "chamber" there are two pores, that could allow the release of the cleaved products of the enzyme, and two "thin density" regions, that could represent transient gates permitting the access of TMD substrates to the active site, curiously resembling the structure of the cytosolic proteasome (Lazarov et al., 2006). Another puzzling issue is mapping the interactions between the various components: a chemical cross-linking study demonstrated close interactions between PS CTF and NTF, PS NTF and Pen-2, PS CTF and Aph-1(aL), Aph-1(aL) and NCT (Fig. 5) (Steiner et al., 2008; Spasic & Annaert, 2008).

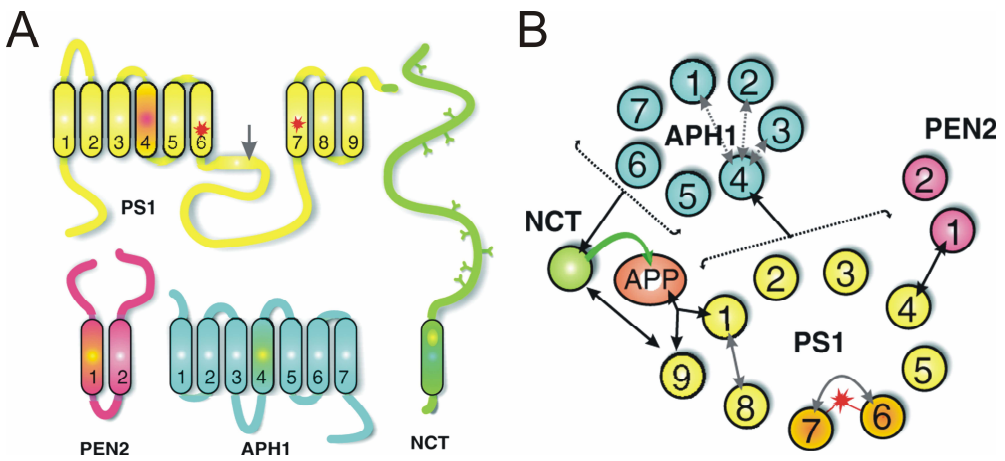

Fig.5: A) The membrane topologies of the four components of the γ-secretase complex. The arrow indicates the presenilinase cleavage site; red asterisks show the position of the catalytic aspartate residues in TMD6 and TMD7 of PS1. Mixed colours indicate intermolecular interaction between TMDs. B) A bird's eye view of the TMDs of PS1 (yellow), NCT (green), Aph-1 (blue) and Pen-2 (red), including the reported (continue) or suggested (dotted) intra- (grey arrows) and intermolecular (black arrows) interactions. Ectodomain interactions of NCT with APP-CTF are indicated with a green arrow, while the red sparkle denotes the catalytic site (modified from Spasic & Annaert, 2008).

It is important to stress that Presenilins, and all the γ-secretase components, were initially thought to be present in the ER and Golgi compartments (Walter et al., 1996) and, only in second instance, also at the level of plasma membrane where the enzyme should be active. This discrepancy became evident quite soon and was called “spatial paradox” (Annaert & De Strooper, 1999). Actually, only a minor fraction of total γ-secretase complexes seems to be active, and this probably account for less than 10 % of the total, and should be located at the plasma membrane or in endosomes, while the rest is present in intracellular membranes but in inactive state (Kaether et al., 2006a and b; Chyung et al., 2005).

The studies on the components of γ-secretase, their interactions and their localization lead the way towards a model for the complex assembly and trafficking (reviewed in Dries & Yu 2008 and in Spasic and Annaert, 2008): all the components are originally synthesized in the ER where they are retained until they undergo the proper incomporation in the complex; once glycosylated, one molecule of NCT binds to one molecule Aph-1, forming the first stable subcomplex; this latter thus binds to PS holoprotein, generating a ternary subcomplex, that subsequently binds to Pen-2 (alternatively, Pen-2 seems to bind to PS holoprotein before the latter binds to the first subcomplex). This stage (NCT+Aph-1+PS holoprotein+Pen-2), however, is transient since the incorporation of Pen-2 results in the endoproteolysis of PS to

its dimeric, active structure. Noteworthy, it has been reported that the complex assembly occurs before or within COPII vesicles, protein transport carriers exiting the ER (Kim et al., 2007). Most of the complexes probably cycle between the ER and the Golgi and only a minor proportion is going through the Golgi where NCT is further post-translationally modified before reaching endosomes and plasma membrane (Fig. 6).

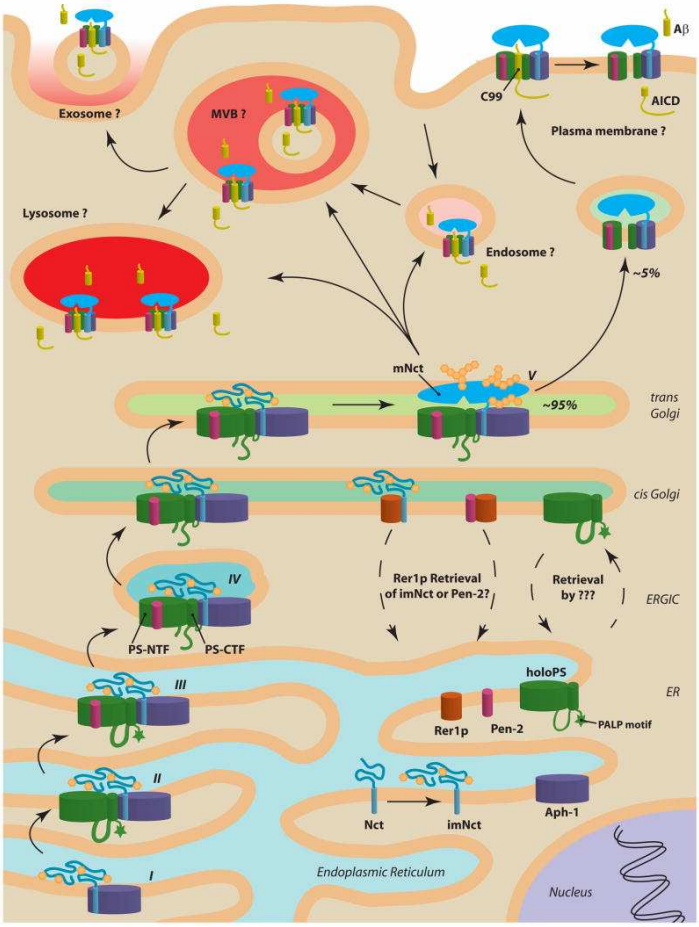

Fig. 6: assembly and trafficking ot the γ-secretase complex (Dries & Yu, 2008)

Presenilin FAD mutations: Aβ and beyond

From a pathological perspective, much interest has been paid to the effects of FAD mutations in PSs on the production of Aβ peptides and, in particular, of the most toxic and fibrillogenic Aβ42. From a mechanistic point of view -increased deposition of amyloid plaques in AD brains (see above), the familial forms linked to mutations on APP gene and the

strong correlation between the duplication of chromosome 21 (Down's Syndrome), on which APP gene is located, and AD (Ertekin-Taner, 2007; Selkoe, 2001)- it was quite obvious to expect that mutations on Aβ-generating enzyme should increase its production, especially the Aβ42 specie.

Actually, this was initially reported: several studies described an increased production of Aβ, mostly Aβ42, peptides in cell models, in cells from patients and in transgenic mice carrying FAD-linked PSs mutations, leading to the notion that FAD mutations in PSs increase the production of Aβ peptides (Xia et al., 1997; Citron et al, 1997; Scheuner et al., 1996; Borchelt et al. 1996).

In the last few years, however, several reports drastically changed this view: although an increased Aβ42/Aβ40 ratio was found for FAD-PSs, it appeared that this was actually due to a reduction in Aβ40 compared to Aβ42, suggesting a total decreased activity of the enzyme in presence of PS-FAD mutants (Florean et al., 2008; Shimojo et al., 2007; Bentahir et al, 2006; Walker et al., 2005). Moreover, this was in agreement with other studies on different γ-secretase substrates and with works showing AD-like neurodegeneration in conditional knock-out mice for PSs (Chen et al., 2008; Bentahir et al., 2006; Saura et al., 2004). Altogether these data made necessary to reconsider the "gain-of-function" hypothesis of FAD mutations in PSs in favor of a "loss-of-function" hypothesis: mutations in PSs, instead of leading to a general increase in Aβ peptides generation, would impair the cleavage efficiency of PSs (not only on APP but also on different substrates), both reducing the total Aβ production and promoting less precise cuts of APP stub with a major generation of Aβ42. Again, this means a shift in Aβ42/Aβ40 ratio towards the more toxic and amyloidogenic specie (as reviewed in De Strooper, 2007 and in Shen & Kelleher, 2007).

Aβ-hypothesis of Alzheimer's Disease is however demonstrating itself as partial and unsatisfactory (Hardy, 2009) and of course the rising of "Presenilin-loss-of-function" theory made it even weaker. At the same time, PSs revealed themselves as complex and pleiotropic proteins, with implications in several and distinct cell life aspects, above all the central and multifaceted calcium homeostasis (Cowburn et al., 2007).

Before portraying an outline of PSs' effect on cellular calcium handling, it would be useful to summarize the key features of this essential second messenger dynamics.

Calcium homeostasis

Calcium (Ca^{2+}) is a key second messenger in cells regulating several different cell functions, from the rapid control of exocytosis and contraction to the regulation of metabolism, or gene transcription or even cell proliferation (Fig. 7). This necessarily requires a highly versatile and refined handling of Ca^{2+} dynamics by the cells (Clapham, 2007; Berridge et al., 2003).

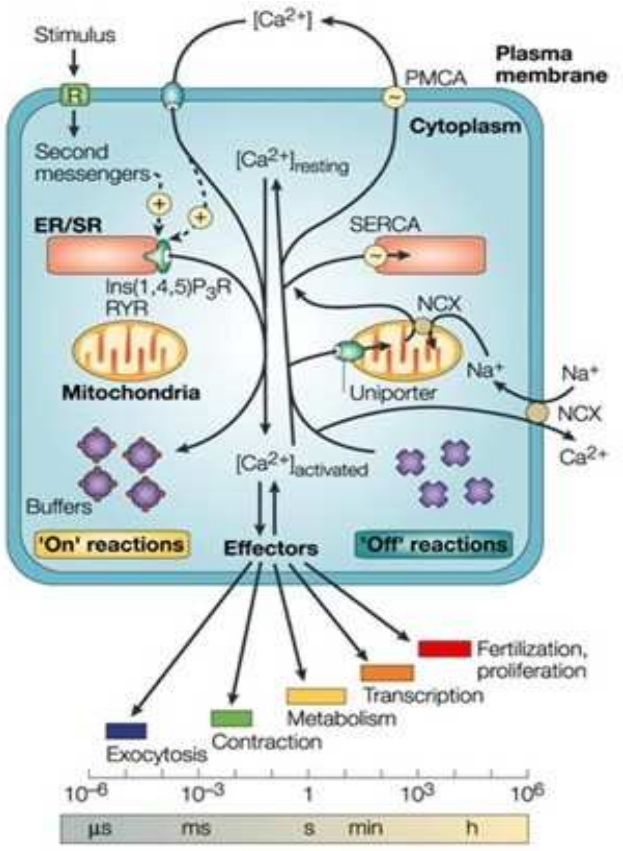

Fig. 7: Ca^{2+} homeostasis and Ca^{2+}-regulated processes (from Berridge et al., 2003)

Many of the processes controlled by Ca^{2+} depend on time-specific and finely integrated changes (rises) in its concentration ($[Ca^{2+}]$) in the cytosolic compartment (Boulware & Marchant, 2008), where several Ca^{2+} sensors are present (see below), and for this reason cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in cells is usually very low, in the submicromolar range. On the contrary, the $[Ca^{2+}]$ in the extracellular media ($[Ca^{2+}]_{ext}$) is relatively high (1-2

mM), as well as in some cell compartments that can behave like "Ca^{2+} stores", first of all the endoplasmic reticulum (ER, see below): actually, extracellular media and intracellular stores are the two sources of Ca^{2+} responsible for its transient rises in the cytosol. Ca^{2+} entry from extracellular space depends on many different plasma-membrane channels that allow the passage of Ca^{2+} ions in response to specific stimuli; internal stores release Ca^{2+} when their Ca^{2+} releasing channels open upon stimulation by intracellular messengers (including Ca^{2+} itself). Of course, Ca^{2+} signals must be properly turned off to ensure the temporal specificity required for controlling cell functions and to avoid detrimental effects due to an excessive stimulation; the "off mechanisms" of Ca^{2+} signals rely on its extrusion from the cytosol to the extracellular space and on its re-accumulation in the same stores capable of its release. The molecular machinery that works to reduce $[Ca^{2+}]_{cyt}$ consists of several pumps, ions exchangers and calcium buffers (Clapham, 2007; Berridge et al., 2003; Pietrobon et al., 1990).

The cytosolic compartment is not a mere space where Ca^{2+} signals are generated and propagated. It contains cell organelles participating in Ca^{2+} homeostasis and is also endowed with multiple proteins capable of binding Ca^{2+}, buffering it and shaping its signals: collectively, it can be called "Ca^{2+} homeostasome" (Schwaller, 2009). Among all the Ca^{2+}-binding proteins, the most popular is probably Calmodulin (CaM), a soluble 15 kDa polypeptide, with four "EF-hands", domains specialized in binding Ca^{2+} formed by two α-helixes and a 12 aa loop; an essential CaM's property is its striking structural change upon Ca^{2+} binding, that leads to the exposure of hydrophobic structures and thus allows the interaction with several proteins, in turn modulated by CaM (Vetter & Leclerc, 2003).

"EF-domains" are shared with a number of Ca^{2+}-binding proteins, usually distinguished in "Ca^{2+} sensors" and " Ca^{2+} buffers": Ca^{2+} sensors are proteins, like CaM itself, that after undergoing large Ca^{2+}-induced conformational changes modulate other intracellular effectors, while Ca^{2+} buffers, like Parvalbumin, Calbindin and Calretinin, are proteins mostly involved in chelating Ca^{2+} ions; however, the distinction between the two groups is starting to blur (Ikura, 1996; Schwaller, 2009).

This high buffering capacity in the cytosolic compartment provides further specificity to Ca^{2+} signalling: limiting Ca^{2+} diffusion rate, together with subcellular compartimentalization, permits a very subtle spatial organization of Ca^{2+} signals, from the tiny and extremely local "microdomains" to more general and massive "waves" (Rizzuto & Pozzan, 2006; Berridge, 2006).

Cellular membranes essentially act as barriers that isolate two compartments within the cell with distinct features, e.g. ionic composition, while plasma membrane (PM) is an essential border that identify the cell as a unit and allow the maintenance of its internal properties and functions. At the same time, thanks to the multitude of receptors and channels on its surface, PM represents the first integration site of different signals converging on the cell from different origins. The most rapid signals that PM provides to the cell are based on ionic movements (mostly from outside to inside the cell), that in turn depend on electrochemical gradients (ensured by membrane itself) and on the opening of specific structures present on the membrane, the ion channels (Clapham, 2007; Rizzuto & Pozzan, 2006).

Ion channels are protein complexes with two essential properties: firstly, upon proper stimulation they undergo a rapid conformational change converting themselves from an impermeable structure to a highly permeable hole through with ions can pass; secondly, each channel is characterized by defined ionic selectivity and conductance.

Thus, channels can be classified according to their ionic selectivity (Ca^{2+} channels, Na^{+} channels, K^{+} channels, Cl^{-} channels) or to the kind of stimulation required for their opening. In excitable cells, an essential role is played by channels regulated by membrane depolarization (Voltage-Operated Channels, VOCs) and by channels gated by the binding of an extracellular ligand (Receptor-Operated Channels, ROCs); other channels can be regulated by intracellular second messengers (Second-Messenger-Operated Channels, SMOCs) or by depletion of intracellular Ca^{2+} stores (Store-Operated Channels, SOCs, see below). (Berridge et al., 2003).

Extracellular ligands do not bind only to ROCs, but can act also on other kinds of receptors, like Receptor-Tyrosin-Kinases (RTKs) and G-protein-coupled Receptors (GPCRs). RTKs initiate phosphorylation cascades inside the cells upon their dimerization and self-phosphorylation, while GPCRs activate the trimeric G-protein to which they are functionally coupled; of particular interest in Ca^{2+} signalling are GPCRs linked to the activation of Phospolipase-Cβ (PLCβ): once activated, this enzyme cleaves phosphatidylinositol-4,5-biphosphate (PIP_2) present in the membrane producing the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglicerol (DAG). As will be discussed hereafter, IP_3 is the main agonist of a receptor (IP_3R) expressed on the membrane of intracellular Ca^{2+} stores and induces its opening with a massive release of Ca^{2+} (see Fig. 10 below). Different mechanisms

than GPCRs can activate other isoforms of PLC and lead to the production on IP_3 (Berridge, 2003).

PM hosts also proteins capable of extruding Ca^{2+} ions to maintain a low cytosolic level or to terminate Ca^{2+} signals: the 130 kDa Plasma-Membrane Ca^{2+}-ATPase (PMCA) is a 10 TMDs protein with high affinity for Ca^{2+} that can exports 1 Ca^{2+} ion upon the hydrolysis of 1 molecule of ATP; it represents an essential housekeeping system to set and maintain the normally low $[Ca^{2+}]_{cyt}$ (Brini, 2009; Streheler et al., 2007). The Na^+/Ca^{2+} exchanger (NCX) is a 110 kDa protein that avails itself of the Na^+ gradient existing between the extracellular environment and the cytosol to extrude Ca^{2+} ions (with a ratio of 3 Na^+ ions for 1 Ca^{2+} ion); however, because of its dependence on Na^+ electrochemical gradient it can revert its operative direction upon PM depolarization and thus cooperate with Ca^{2+} VOCs allowing cell Ca^{2+} entry (Blaustein & Lederer, 1999).

Inside the cell, a multitude of subcellular structures are involved in Ca^{2+} handling. The major intracellular Ca^{2+} store is the ER, because of its high capacity and its huge volume, but many other organelles can participate in controlling Ca^{2+} homeostasis and/or are influenced by changes in $[Ca^{2+}]$, like mitochondria, Golgi apparatus, nucleus, (Bootman et al., 2009; Rizzuto & Pozzan, 2006; Michelangeli et al., 2005), with recent new insight into lysosomes, endosomes and peroxisomes' contribution to Ca^{2+} homeostasis (Calcraft et al., 2009; Drago et al., 2008; Lasorsa et al., 2008). As will be discussed hereafter, particular interest is given to the functional interaction between ER and mitochondria, key organelles in determining cell activity highly sensitive to Ca^{2+} dynamics (Fig. 8).

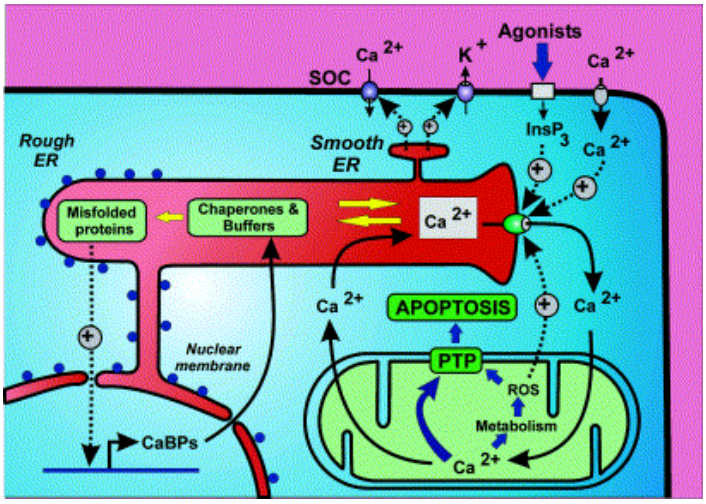

Fig. 8: ER and mitochondria interplay in Ca^{2+} homeostasis (see below for details).

Key players in calcium homeostasis I: Endoplasmic Reticulum

Endoplasmic Reticulum (ER) exerts many functions in the cell, including translocation of proteins across its membrane, integration of proteins into the membranes, folding and modification of proteins in its lumen, phospholipids and steroids synthesis and, last but not least, Ca^{2+} storage and release upon stimulation. Although it has been demonstrated that ER is a single membrane network with a continuous intralumenal space, functional heterogeneity can isolate internal domains; an obvious distinction is between nuclear envelope (or "nuclear ER"), rough ER (ribosome-studded ER, involved in protein synthesis) and smooth ER, that can have different functions in different cell types, firstly Ca^{2+} storage (Fig. 9; Rizzuto & Pozzan, 2006; Voeltz et al., 2002; Berridge, 2002).

Ca^{2+} is accumulated in the ER through the Sarco/Endoplasmatic Ca^{2+}-ATPase (SERCA), a 110 kDa enzyme that moves Ca^{2+} ions from the cytosol into the lumen of ER against its chemical gradient; it possesses a large cytosolic domain, a transmembrane domain formed by 10 α-helixes (an additional small C-terminal TM tail is present in isoform 2b) and small lumenal loops. At resting $[Ca^{2+}]_{cyt}$, SERCA activity is low but enough to compensate the continuous leak of Ca^{2+} across the ER membrane (see below). Upon a rise in $[Ca^{2+}]_{cyt}$ (and a decrease in $[Ca^{2+}]_{ER}$), its activity quickly increases, providing a major tool to terminate Ca^{2+} signals and refill ER store (Meldolesi, 2001; SERCA structure and mechanism of action are extensively described in Toyoshima, 2009).

Once inside ER, Ca^{2+} is held in the lumen of the organelle by a number of Ca^{2+}-binding proteins, most of them acting both as buffers or as protein chaperones, like Calreticulin (CRT), GRP94, GRP78, Calnexin and Calsequestrin; their large capacity for Ca^{2+} ensures the high $[Ca^{2+}]_{ER}$ (usually estimated around 500 μM) while their relatively low affinity for the ion grants its rapid diffusion through the organelle and its prompt release upon the opening of its releasing channels (Berridge, 2002; Meldolesi, 2001).

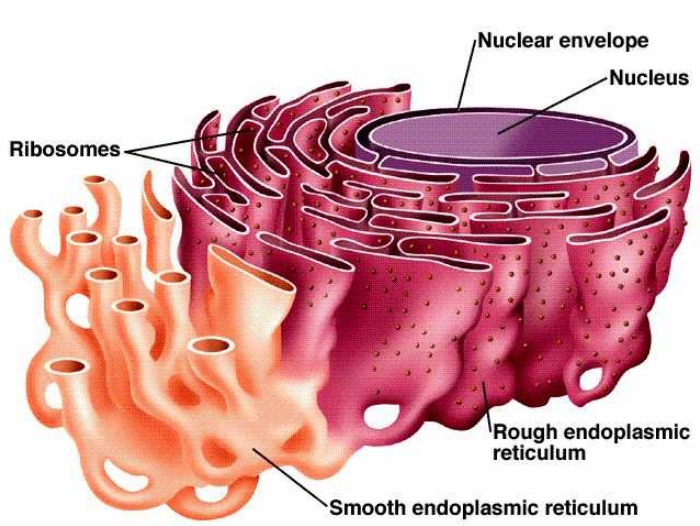

Fig. 9: ER structure: ER is a single membrane network organized in tubules and cisternae, and can be divided in nuclear envelope, rough ER and smooth ER.

The two Ca^{2+}-releasing channels within the ER membrane are inositol-1,4,5-triphosphate Receptors (IP_3Rs) and Ryanodine Receptors (RyRs) with a shared general structure, being high molecular weight tetrameric receptor-channels. IP_3R isoforms (IP_3R-1, 2, 3) are encoded by three different genes but their sequences show high homology; each one contains approximately 2700 aa with a predicted molecular mass of 300 kDa (around 260 kDa according to electrophoretic motility); the three isoforms can have overlapping expression patterns and seem to be functionally distinct, albeit this is not completely defined; further functional complexity can arise from the fact that the three isoforms can be co-assembled generating hetero-tetramers. The main agonist of the IP_3Rs is IP_3 itself, generated upon the activation of PLCs (Fig. 10); the receptor is subjected of regulation by several mechanisms, among which the most intriguing is Ca^{2+} itself: the IP_3R regulation by $[Ca^{2+}]_{cyt}$ is biphasic, since a modest $[Ca^{2+}]_{cyt}$ favours the maximal activation of the receptor by IP_3, but high

$[Ca^{2+}]_{cyt}$ inhibits it; in turn, IP_3 can sensitize the receptor to Ca^{2+}, making the receptor as a "coordination sensor" capable of amplifying intracellular signals (Berridge, 2002 and 1998, Meldolesi, 2001; IP_3R structure and regulation is extensively reviewed in Foskett et al., 2007).

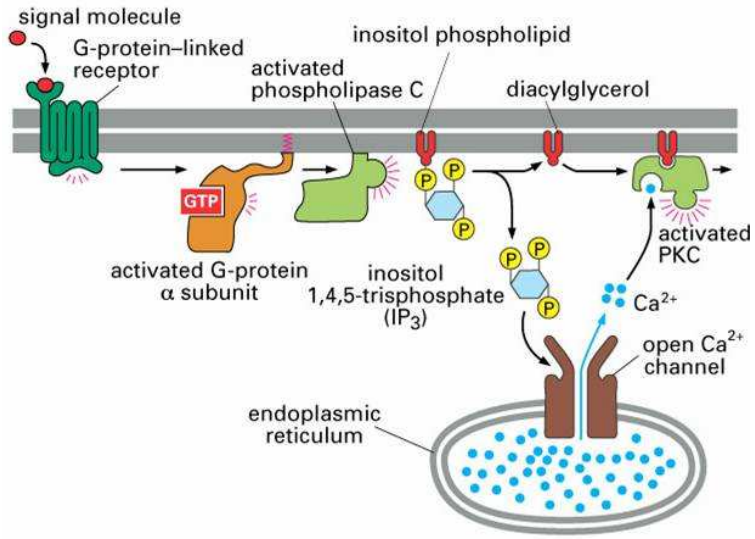

Fig. 10: IP_3Rs opening by IP_3 produced upon the activation of a GPCR and the consequent stimulation of PLC.

The huge RyRs (MW around 500 kDa) take their name from the alkaloid Ryandine, probably their most popular antagonist; as for IP_3Rs, three different isoforms exist in mammalian cells (RyR-1, 2, 3) but, also in this case, the functional implication of this diversity is not still clear. The main physiological activator of RyRs is Ca^{2+}, a phenomenon described as "Ca^{2+}-Induced-Ca^{2+}-Release" (CICR, reported also for IP_3Rs): depending on their localization, RyRs can be gated by a local $[Ca^{2+}]_{cyt}$ increase due to Ca^{2+} entry from PM channels or to Ca^{2+} release from intracellular stores, in particular form IP_3Rs in the ER; thus the RyR can act as positive modulator of Ca^{2+} signals, enhancing their potency and/or sustaining their propagation (Fig.11). Again, the receptor Ca^{2+} regulation is biphasic with high $[Ca^{2+}]_{cyt}$ inhibiting the channel (Meldolesi, 2001; Berridge, 1998; RyRs are extensively reviewed in Fill & Copello, 2002).

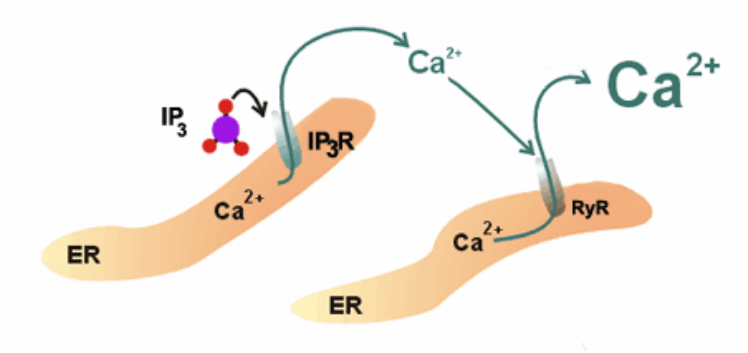

Fig. 11: enhancement and propagation of Ca^{2+} signals by Ca^{2+}-Induced-Ca^{2+}-Release.

ER however can increase $[Ca^{2+}]_{cyt}$ not only by releasing the ion by opening its Ca^{2+} channels, but also by a second, indirect way, named "Capacitative Ca^{2+} Entry" (CCE): a reduction of $[Ca^{2+}]_{ER}$ induced by several stimuli is sensed by the above mentioned SOCs and leads to their opening and to the entry of Ca^{2+} from the extracellular space; this Ca^{2+} influx can amplify the ongoing Ca^{2+} signal or be uptaken by SERCAs to refill the stores. The mechanism of CCE activation and the identity of the SOC responsible for it have been largely debated (Venkatachalam et al, 2002). In 2005 Liou and collegues (Liou et al., 2005) by siRNAs screening revealed the identity of the $[Ca^{2+}]_{ER}$ sensor(s), the STromal Interaction Molecule, STIM 1 (and with some distinctions STIM 2), which has one TMD and a single EF domain exposed in the ER lumen, sensing $[Ca^{2+}]_{ER}$. Upon a decrease in $[Ca^{2+}]_{ER}$ STIM molecules oligomerize in defined structures called "*punctae*" in close proximity with PM. Soon after, it became clear that Orai-1 is the essential pore subunit forming the SOCs (Prakriya et al., 2006) and that STIM-1 *punctae* induce the oligomerization of Orai-1 channels in the membrane, thus activating CCE (Luik et al., 2008; STIM-1 and Orai-1 interaction in CCE activation is reviewed in Cahalan, 2009, Fig. 12).

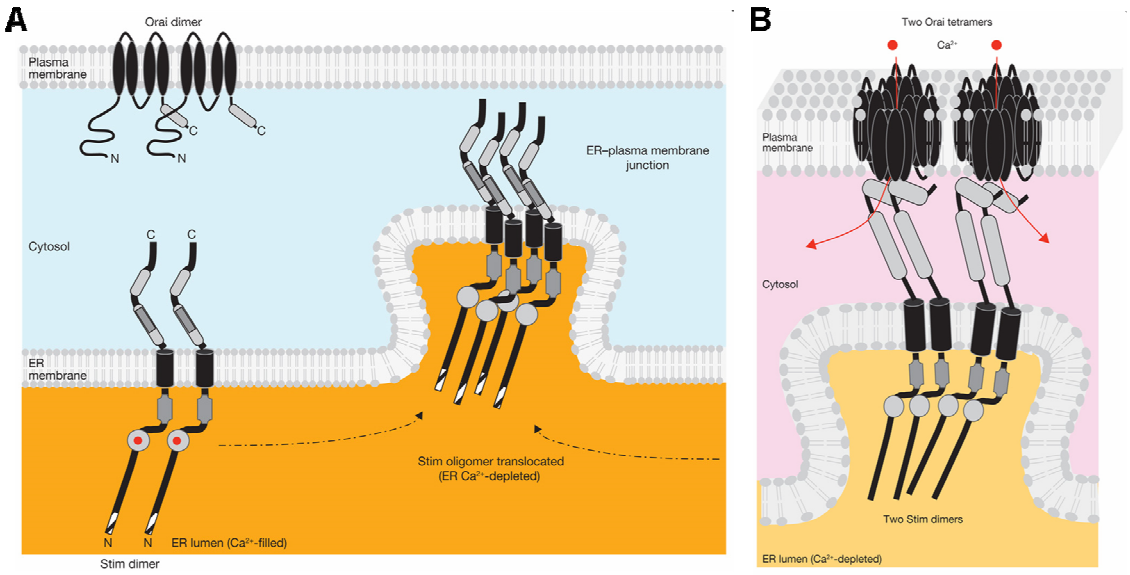

Fig. 12: ER Ca^{2+} depletion induces STIM-1 *punctae* formation, Orai-1 clustering on plasma membrane and CCE activation (modified from Cahalan, 2009).

A feature of the ER Ca^{2+} handling that still lacks a complete understanding is the molecular nature of the Ca^{2+} leak across its membrane. ER filling by SERCA is continuously counteracted by a constitutive leak of ions (and *vice versa*). This process is quite slow (ranging form values around 20 to 200 μM/min depending on the cell type under investigation), in particular compared to the massive and fast depletion that occurred by activation of the Ca^{2+}-releasing channels. Different molecules have been proposed as possible mediators of the ER Ca^{2+} leak: the Ca^{2+}-releasing channels themselves (RyRs and IP_3Rs), the SERCA, the Ribosome-Translocon-Complex (the molecular machinery that transports newly synthesized protein chains across ER membrane), the anti-apoptotic protein Bcl-2 and, recently, Presenilins (Tu et al., 2006;Van Coppenolle et al, 2004; Camello et al., 2002; Pinton et al., 2000).

ER-mitochondria interaction: the Mitochondria-Associated-Membranes

The functional coupling between ER and mitochondria has emerged as an essential feature of the cell life and new specialized domains of the ER membrane came to the attention of the researchers because of their close interaction with mitochondria: the Mitochondria-Associate-Membranes (MAMs, Rusiñol et al., 1994). These interactions between ER and Mitochondria,

by definition the "powerhouse" of the cell and a major Ca^{2+} buffering organelle (a description of mitochondrial Ca^{2+} handling will be given hereafter), are correlated with several cellular functions, like lipid transport, energy metabolism, cell survival and, of course, Ca^{2+} signalling (Giorgi et al., 2009; Hayashi et al., 2009; Pizzo & Pozzan, 2007, Fig 13). Evidence suggests that the link between ER and mitochondrial membrane is formed by tether proteins that can have various length, from 10 to 25 nm; the presence of this tethers seems to be required for the proper Ca^{2+} shuttling between ER and mitochondria and tightening the link between the two organelles with an exogenous linker increases the probability of Ca^{2+} uptake and of toxic Ca^{2+} overload in mitochondria (see below for details, Csordás et al., 2006).

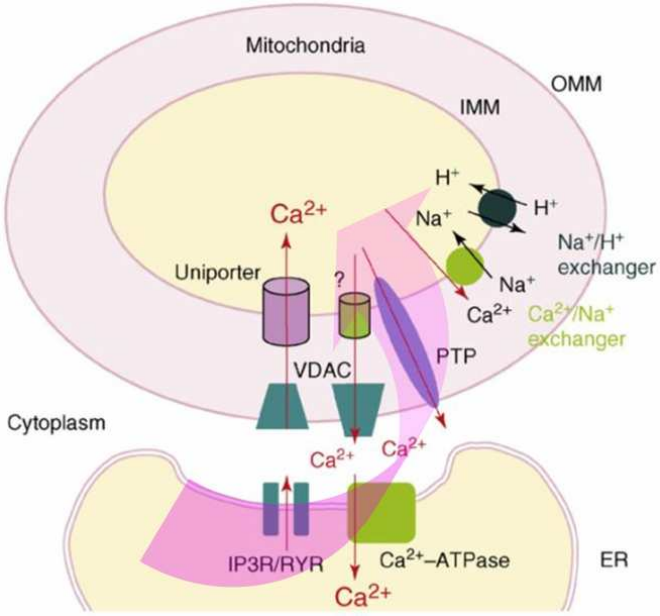

Fig. 13: Major Ca^{2+} transporters and channels at the mitochondrion/ER interface: Ca^{2+} released from ER across IP3Rs/RyRs is uptaken into mitochondria via VDAC and Ca^{2+} uniporters (see text for details, from Hayashi et al., 2009).

Great interest is being given to the proteins exclusively, or preferentially, expressed at the MAMs level: the list is everyday longer but, among all, it is possible to mention metabolic enzymes, like PtdSer synthase, ion channels or transporter, such as SERCA and IP_3Rs (IP_3R-3 in particular, Mendes et al., 2005), molecular chaperones, like CRT, Calnexin and the recently discovered Sigma-1 Receptor (Hayashi & Su., 2007), and many others (see Hayashi et al., 2009 for a comprehensive review); notably, also Presenilins have been recently reported to be enriched in MAMs (Area-Gomez et al., 2009). The identity of the possible tethers is under

investigation: grp75 chaperone has been demonstrated to link IP_3Rs and the mitochondrial Voltage Dependent Anion Channel VDAC (see below; Szabadkai et al., 2006) and a mitochondrial fusion protein, Mitofusin 2 (Mfn-2), has been shown to tether ER to mitochondria (de Brito & Scorrano, 2008).

Key players in calcium homeostasis II: mitochondria

Mitochondria are highly dynamics organelles formed by two membranes, that identify four regions: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM) and the mitochondrial matrix (Fig. 14).

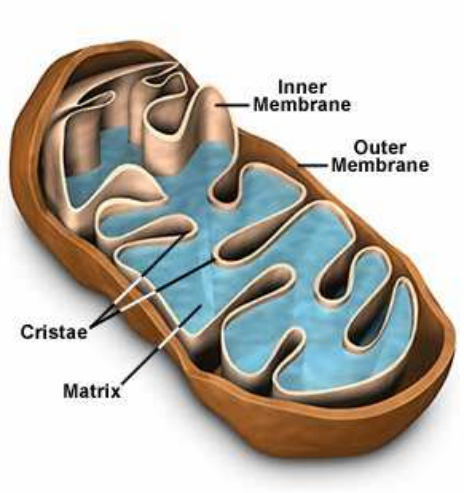

Fig. 14: mitochondrial structure: mitocondria are composed by an outer membrane (OMM) and inner membrane (IMM) that separate two compartments, the intramembrane space (IMS) and the mitochondrial matrix.

It is well know their essential role in cell metabolism and in energy production, but this parallels the fact that they are key players in Ca^{2+} homeostasis. The OMM can be easily crossed by Ca^{2+}, also with the support of the above mentioned VDAC, while the cation needs a still undefined "Ca^{2+} Uniporter" to enter the matrix (Giorgi et al., 2009). Mitochondrial Ca^{2+} entry is driven by the negative potential across the IMM (up to -180 mV in polarized mitochondria), leading to a very high [Ca^{2+}] (above 100 μM) within the mitochondrial matrix, via activation of the Ca^{2+} Uniporter requiring relatively high $[Ca^{2+}]_{cyt}$ because of its low Ca^{2+}affinity, estimated around 10 μM. This means that fast mitochondrial Ca^{2+} uptake needs

high $[Ca^{2+}]_{cyt}$ that are not seen in the bulk cytoplasm but can be reached locally in proximity of the sites of ER Ca^{2+}-releasing channels or PM Ca^{2+} channels, commonly referred as "high Ca^{2+} microdomains" (Rizzuto & Pozzan, 2006).

The outward transport of Ca^{2+} from mitochondria is mediated by two antiporters (Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters). Another important mitochondrial protein is the mitochondrial Permeability Transition Pore that opens upon mitochondria Ca^{2+} overload or oxidative stress, leading to mitochondrial matrix swelling, collapse of mitochondrial membrane potential, release of pro-apoptotic factor, like Cytochrome C and thus to cell death (Giacomello et al., 2007; Rizzuto & Pozzan, 2006). It is important to stress that the intimate relation between mitochondria and Ca^{2+} controls cell metabolism and energy production - since some mitochondrial key enzymes (pyruvate-, α-ketoglutarate- and isocitrate-dehydrogenases) and some metabolite transporters are Ca^{2+} sensitive-, the shaping of cytosolic signals and Ca^{2+} oscillation (see Ishii et al., 2006) and, lastly, cell fate (Pinton et al., 2008; Giacomello et al., 2007; Rizzuto & Pozzan, 2006).

The high motility of mitochondria refers to the dynamic shaping of the organelles and to their transport and localization inside the cell; these phenomema deeply influence their interaction with the ER. The current view of mitochondria is no more that of relatively small, isolated vescicular structure; rather, they are seen as a network of organelles organized in a dynamic way (Pizzo & Pozzan, 2007). Mitochondrial morphology can be alternatively elongated or shortened and several proteins can control the fission/fusion balance between the single organelles (in particular, the GTPases Drp and Mfn1/2 are involved in fission and fusion respectively, and OPA1 seems to be involved in mitochondrial fusion, Dimmer & Scorrano, 2006; Chen & Chan, 2005).

Mitochondrial transport can use two different tracks in cells: the first one is represented by microtubules along which mitochondria can be moved bidirectionally by kinesins and dyneins, providing the most rapid and longest form of mitochondrial transport; the second one is represented by actin microfilaments, on which the organelles are driven by myosins, mostly committed with local adjustment of mitochondria position (Boldogh & Pon, 2007, Frederick & Shaw, 2007).

Importantly, among the different factors controlling mitochondrial movement, Ca^{2+} emerged as a key modulator, in particular after the discovery of the Ca^{2+}-dependent kinesin-mediated mitochondria motility, based on the interaction between the Ca^{2+}-sensitive

mitochondrial GTPase Miro, the adaptor Milton and Kinesin-1 (Wang & Schwarz, 2009; Liu & Hajnóczky, 2009).

Calcium measurements in living cells

Because of the central Ca^{2+} role in cell physiology, the study of its homeostasis in living cells has been – and actually is still - object of great interest. Such a study requires tools capable of measuring Ca^{2+} dynamics with spatial and temporal accuracy and adequate sensitivity, considering the fact that Ca^{2+} concentration can vary strikingly inside the cells, depending on the compartment considered or on the state (resting/stimulated) of the cell itself (Rudolf et al., 2003).

Ca^{2+}-measuring toolkit comprises synthetic and protein-based probes: synthetic probes are fluorescent compounds that can be loaded in the cells and used for measurements, like Fura-2, Fluo-3, Indo-2; these polycarboxylate-based indicators, originally devised by R.Y. Tsien (Tsien, 1980), are based on EGTA Ca^{2+}-chelator structure and on dye's Ca^{2+}-induced structural changes, leading to a change in the spectral emission and/or excitation properties of the probe (Rudolf et al., 2003).

The second type of Ca^{2+} probes is a "gift from sea deep": between 1960s and 1970s the studies about the phenomenon of bioluminescence on the marine jellyfish *Aequorea victoria* by Shimomura and Johnson led to the identification of two proteins, Aequorin (Aeq, Shimomura et al., 1962) and Green Fluorescent Protein (GFP, Morise et al., 1974): both, in a few years, became extremely relevant for the research in the field of Ca^{2+} homeostasis and, more generally, of cell biology (Rudolf et al., 2003).

Aeq is a Ca^{2+} sensitive photoprotein composed by a 21 kDa apoprotein and a hydrophobic prosthetic group, called Coelenterazine; it contains three high affinity Ca^{2+}-binding sites and, upon binding the ions, the covalent link between the prosthetic group and the apoprotein is quickly broken with the emission of one photon, as an irreversible reaction; importantly, the rate of photons emission depends on the $[Ca^{2+}]$ (Fig. 15). Initially, after Aeq discovery, the protein was extract from jellyfish and microinjected in cells; later, the cloning of its cDNA allowed its recombinant cell expression in a simple way (requiring the exogenous addition of the cell permeant Coelenterazine to obtain functional holoprotein) and its genetic modification, by adding targeting sequences for its specific intracellular localization; for this

purpose, since some cell compartments are endowed with high [Ca^{2+}], mutant Aeq with lower affinity for Ca^{2+} (and thus with lower consumption rate) have been created; alternatively, the employment of Aeq in high [Ca^{2+}] compartments has been improved by using modified prosthetic groups (e.g. Coelenterazine N) or by substituting Ca^{2+} with the surrogate Sr^{2+} (reviewed in Brini, 2008; Chiesa et al., 2001).

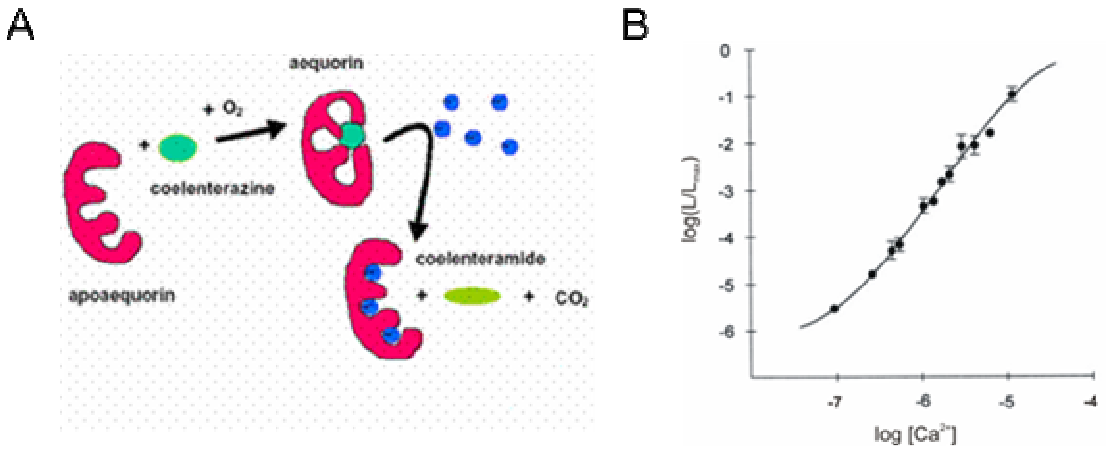

Fig. 15: A) Schematic representation of aequorin reaction upon binding Ca^{2+}; B) calibration curve of aequorin correlating the logarithm of the light emitted to the logarithm of Ca^{2+} concentration.

The wide variety of Aeq localizations made it a useful tool for studying Ca^{2+} dynamics: Aeq is *per se* a cytoplasmatic protein but the fusion of its sequence with the mitochondrial pro-sequence of the VIII subunit of respiratory chain Cytochrome C Oxidase permitted its localization in the mitochondrial matrix (Rizzuto et al., 1992); ER-Aeq was obtained in two different ways: Kendall and collegues (Kendall et al., 1994) added a CRT-derived ER insertion signal and the KDEL targeting sequence to Aeq, while Montero and collegues fused Aeq with the N-terminal region of the immunoglobulin heavy chain, containing the leader sequence (for ER insertion) and the CH1 domain (for retrieval from trafficking to other compartments) (Montero et al, 1995). Further Aeq localizations have been achieved with various strategies, e.g. mitochondrial intramembrane space, Golgi apparatus, nucleus, sub-plasmalemmal region and peroxisomes (Brini, 2008; Chiesa et al., 2001).

Considering the achievements of recombinant Aeq targeting and the possibility of a quick calibration of photons collected during experiments in [Ca^{2+}] through an algorithm, Aeq-based approach to study Ca^{2+} dynamics is extremely versatile and trustworthy; however its major weakness stands in the low amount of light emitted by the photoprotein: this means that

it is quite difficult to obtain results at the single cell level by using Aeq-probes, and thus it is mostly employed for cell population analysis (Brini, 2008).

The second "gift" from *A. victoria* was GFP: the Green Fluorescent Protein became an essential tool in cell biology soon after its cloning and the discovery that it retains its fluorescent properties also when expressed in eukaryotic cell, starting with its employment as a genetic marker (Chalfie et al., 1994). It didn't take too long before mutants with improved features or spectral shifts were discovered (Chalfie, 1995; Heims et al., 1994).

An intriguing application of GFP and its mutants was the generation of protein-based probes to monitor several cell functions, for example Ca^{2+} dynamics. Several approach to create GFP-based Ca^{2+}-probes were conceived, all based on the Ca^{2+}-sensor CaM, because of its sensitivity for Ca^{2+} ions and for its structural change upon binding it: the change in CaM conformation upon Ca^{2+} binding would be translated into a change in the spectral properties of the GFP(s) fluorescence, thus allowing to sense variation in $[Ca^{2+}]$. Indeed the three different GFP-CaM-based sensors available are called "pericams", "camgaroo" and "cameleons" (Rudolf et al., 2003). However, among the three, Cameleons are the most widely diffused.

It was again the group of R.Y. Tsien that started the realization of this latter Ca^{2+} probe (Miyawaki et al., 1997): it is based on two different GFP, the Blue-shifted mutant Fluorescent Protein (BFP) and the Green Fluorescent Protein (GFP), linked by CaM and its binding peptide M13; upon binding Ca^{2+} ions, CaM wraps around the M13 peptide bringing the two fluorophores in close proximity and increasing the fluorescence resonance energy transfer (FRET, see below) between them (Fig. 16). As for Aeq-based probes, the possibility of selective targeting in cell compartments and of changing the Ca^{2+}-affinity of the probe became immediately clear. However, even after the substitution of BFP and GFP with the enhanced Cyan and Yellow mutants ("Yellow Cameleons"), several limitations were still evident, in particular the pH sensitivity of YFP and the interference of endogenous CaM or CaM-binding proteins: further improved Yellow Cameleon (Improved YC, named YC2-4), with reduced pH sensitivity, were thus developed (Miyawaki et al., 1999). Since the problem of the perturbation by the large excess of native CaM was not still solved, Cameleons have been recently re-designed obtaining probes less affected by intracellular environment, with different Ca^{2+} affinity and with a broader dynamic range, allowing better analysis of Ca^{2+}

dynamics in different compartments (named D1-4cpv, Palmer et al., 2006). Indeed, some of this "new Cameleons" have been employed in this work.

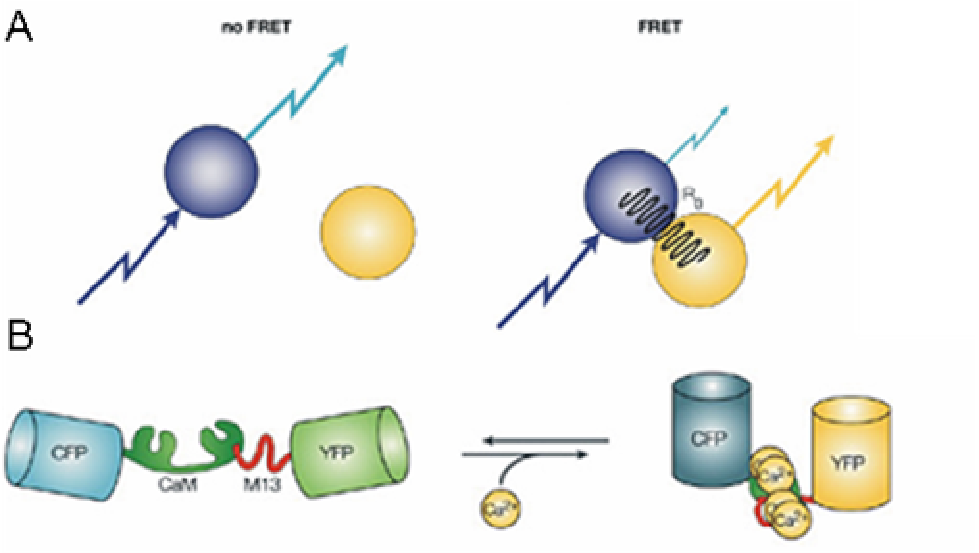

Fig. 16: A) Schematic representation of Fluorescence Resonance Energy Transfer (FRET) phenomenon; B) in Cameleon probes, the conformational change induced by Ca^{2+} in CaM molecule brings CFP and YFP in proximity and allow FRET between the two fluorophores (see text for details, modified from Rudolf et al., 2003)

Fluorescence Resonance Energy Transfer (FRET) can be simply described as the non-radiative energy transfer between a donor cromophore and an acceptor cromophore when their emission (for donor) and excitation (for acceptor) spectra overlap and when the two molecules are close enough (2-7 nm) and in the adequate relative orientation. Considering Cameleons probes, when FRET does not occur (low $[Ca^{2+}]$) CFP is excited at 440 nm and emits at 480 nm, while YFP is substantially unaffected; when $[Ca^{2+}]$ rises, the conformational change, due to CaM, brings closer CFP and YFP; excited CFP transfers its energy to YFP, that emits at 530 nm. Moreover, this means that a rise in $[Ca^{2+}]$ can be observed both as an increase in YFP (530 nm) emission and as a decrease in CFP (480 nm) emission; better, the two signals can be compared obtaining a ratio between F530/F480, improving much more the reliability of the readout, since a rise in $[Ca^{2+}]$ will lead to a magnified increase in F530/F480 ratio and *vice versa*. Indeed, Cameleon FRET-based Ca^{2+} probes can be target to several cell compartments and efficiently used to monitor Ca^{2+} dynamics at the single cells level (Rudolf et al., 2003, Fig. 16).

Presenilins and calcium

It is evident that Ca^{2+} plays an essential role in cell life and that any perturbation in its handling mechanisms can have detrimental effects on cell homeostasis. This is particularly true in neurodegeneration where Ca^{2+} has been demonstrated to exert a central role (Bezprozvanny & Mattson, 2008; Mattson, 2007). It is since middle 80s that Ca^{2+} has been reported to be perturbed in sporadic AD patients, albeit in contrasting ways (Tatebayashi et al., 1995; Peterson et al., 1986 and 1988).

The link between AD and Ca^{2+} dysregulation became more evident with the studies on FAD mutations, especially in PSs, that have revealed strong – and controversial – effects on Ca^{2+} signalling (Bezprozvanny & Mattson, 2008; Bojarski et al., 2007).

The most diffuse (but not universally accepted) hypothesis on FAD-linked Ca^{2+} dysregulation is the "Ca^{2+} overload" hypothesis, stating that FAD-PSs should lead to an increased Ca^{2+} content in cell stores and thus to an exaggerated Ca^{2+} release upon stimulation. This was demonstrated in several works based on cell lines transiently expressing mutant PS (in particular PS1) or primary cortical neurons, or brain slices, from transgenic mice, mostly by evaluating cytosolic Ca^{2+} release with cytosolic fluorescent dyes (Schneider et al., 2001; Leissring et al. 2000 and 1999; Furukawa et al., 1998; Guo et al., 1996). However, this was not confirmed by all groups, in particular when considering FAD-PS2 and employing probes directly targeted to the ER lumen, like ER-Aeq (Giacomello et al., 2005; Zatti et al., 2004).

In recent years, the analysis of the role by PSs (and by FAD-PSs) on Ca^{2+} homeostasis received greater attention, again with some controversies (Fig. 18, see below).

PSs were shown to form a low conductance Ca^{2+}-leak channel in the ER membrane (putatively accounting for the 80 % of the total ER Ca^{2+} leak), as demonstrated by experiments with lipidic bilayers fused with microsomes containing PSs; thus, PSs would ensure the physiological $[Ca^{2+}]_{ER}$ preventing its overload; the permeability of the channel would be impaired by FAD mutations, leading to an increase in $[Ca^{2+}]_{ER}$ and thus to a bigger cytosolic release upon stimulation (Fig. 17; Nelson et al., 2007; Tu et al., 2006).

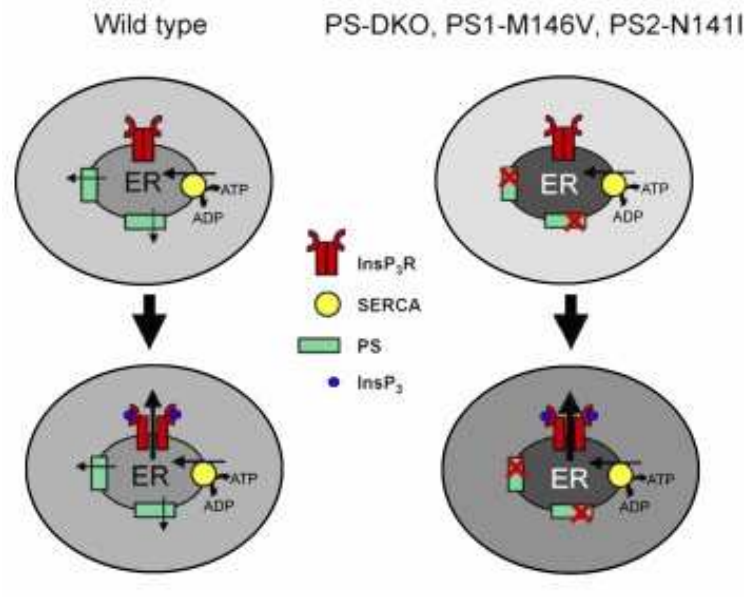

Fig. 17: model proposed by Bezprozvanny and colleagues (Tu et al. 2006) for ER Ca^{2+} overload induced by FAD-PSs; green boxes represent PSs, that in their wt form would allow the Ca^{2+} leak across ER membrane, while those bearing FAD mutations would cause an excessive Ca^{2+} loading of ER.

More recently, PSs have been shown to interact physically with the IP_3Rs and to modulate their gating, with FAD-PSs enhancing the gating and sensitizing the IP_3Rs to lower concentration of agonist; again, the final effect is an exaggerated Ca^{2+} release although in the presence of a slightly decreased $[Ca^{2+}]_{ER}$ (Cheung et al., 2008). Thus, FAD-linked PS mutants do not necessarily induce a Ca^{2+} overload but rather confer a Ca^{2+} hyper-excitability.

Between the different molecular targets proposed to explain the FAD-PSs linked Ca^{2+} alterations, dysregulated IP_3 signaling was also suggested by previous works (Stutzmann et al., 2004), as well as an increased activity of the other intracellular Ca^{2+} channel, the RyRs. In particular, the N-terminal fragments of PS1, and also PS2, were shown to potentiate RyR single channel activity (Hayrapetyan et al., 2008; Rybalchenko et al., 2008). Upregulated RyRs were also proposed as possible mediators of FAD-PS1 action on Ca^{2+} homeostasis (Stutzmann et al., 2007 and 2006; Smith et al., 2005) and, recently, it has been demonstrated that RyR dysregulation is an early alteration preceding the classical pathological symptoms in a triple transgenic mouse AD model expressing pathological mutations in PS1, APP and Tau (Chakroborty et al., 2009).

Also the SERCA molecule has been proposed as a target of PSs: La Ferla and colleagues (Green et al., 2008) showed that the ATPase is regulated by PSs (in particular PS2) and

hyper-activated by FAD-PS1, as observed by evaluating the rate of cytosolic Ca^{2+} rise clearance; they showed also that the suggested enhanced SERCA-dependent ER Ca^{2+} uptake in FAD-PS1 cells leads to an increased IP_3-mediated Ca^{2+}-liberation.

On the other side, fura-2 experiments on FAD human fibroblasts and control cells, as well as on primary mouse cortical neurons over-expressing FAD-PS1/2, demonstrated a reduced Ca^{2+} release from intracellular stores. Moreover, by employing ER- and Golgi-targeted aequorins, to directly evaluate the $[Ca^{2+}]$ within these stores, in different transiently transfected cell models, it was clearly showed a marked reduction in PS2- and, at lower extent, in PS1-transfected cells (Zatti et al., 2006).

On this line, recent data demonstrated a Ca^{2+} deficit in neurons from *D. melanogaster* expressing FAD-PS1/2 compared to control neurons, an effect that seems to require CaM (Michno et al., 2009).

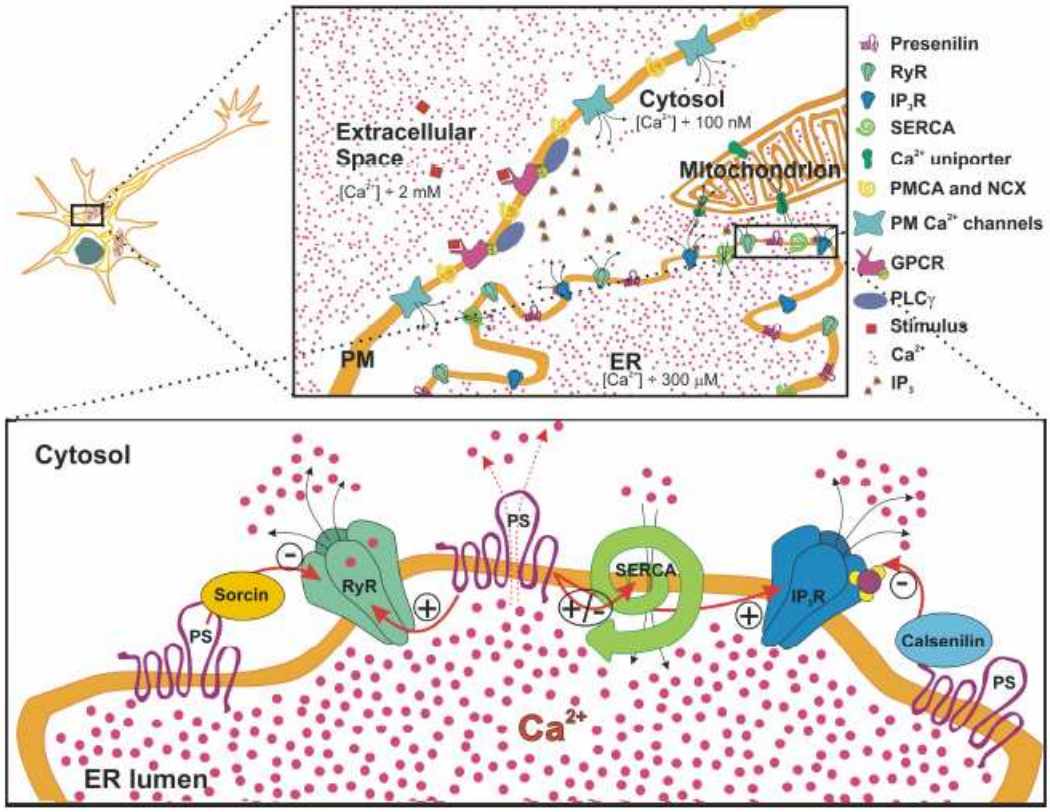

Fig. 18: Complex effects of PSs on Ca^{2+} homeostasis: PSs have been proposed to form a Ca^{2+} leak channel *per se* and to modulate RyRs, IP_3Rs and SERCA (see text for details, Zampese et al., *in press*).

The scenario is complex. It is noteworthy the fact that also data comparing cell lines devoid of endogenous PSs (PS1/PS2 DKO) with wt cells are not homogeneous, since different groups reported very different effects in cells lacking PSs (e.g. Tu et al., 2006; Kasri et al., 2006), indicating that, probably, PSs role on cell signalling and Ca^{2+} homeostasis is extremely multifaceted (Fig. 18).

Aim of this work

The present work started from a previous project in the lab, showing a strong reduction in $[Ca^{2+}]_{ER}$ (and $[Ca^{2+}]_{Golgi}$) in cells expressing FAD-PS2, and was aimed at understanding the possible mechanism(s) behind the described phenomenon.

In particular, it focused on:

- the structure of PS2 (dimeric "mature" or full length "immature" structure) required for reducing $[Ca^{2+}]_{ER}$; as discussed in the following sections, we demonstrated that the full length structure of PS2 is necessary for its effect on ER $[Ca^{2+}]$.
- the role of the different ER Ca^{2+} efflux pathways in the PS2-induced reduction in ER Ca^{2+} levels, e.g. RyRs, IP_3Rs and the Ribosome-Translocon-Complex (RTC); our data evidenced a modest but significant contribution of RyRs and IP_3R in PS2 effect on $[Ca^{2+}]_{ER}$.

Parallel studies on SERCA were conducted in the lab and altogether these data were accepted for publication (Brunello et al., 2009; Zampese et al., *in press*).

The final part of the work focuses the analysis of PS2 effects on cell Ca^{2+} homeostasis considering the interaction between ER and mitochondria. Since PS2 expression reduces $[Ca^{2+}]_{ER,}$ and thus the Ca^{2+} released in cytosol and sensed by mitochondria, we expected that mitochondrial Ca^{2+} uptake would have reflected or, better, enhanced the reduction, because of the specific properties of mitochondrial Ca^{2+} handling that make them very sensitive to small changes in cytosolic Ca^{2+} release.

Actually, we found that mitochondria in (FAD)-PS2 expressing cells do not enhance $[Ca^{2+}]_{ER}$ reduction, but rather have a favoured Ca^{2+} uptake. By investigating the mechanisms behind this phenomenon, we show that (FAD)-PS2s seem to modulate positively ER-mitochondria coupling, thus facilitating mitochondrial Ca^{2+} uptake upon release from the ER.

RESULTS

PS2-T122R overexpression reduces $[Ca^{2+}]_{ER}$ in different cell models

The expression of different PS2 FAD-linked mutants as well as the wt form was shown to decrease the Ca^{2+} content of intracellular stores (Zatti et al., 2006); the molecular nature and the mechanism(s) of this PS2 dependent effect were however not defined.

For this purpose, the effect of FAD-PS2 (the T122R mutation) was checked in several cell models: HeLa cells, SH-SY5Y cells, wt and PS1/PS2 double knock out (DKO, devoid of endogenous presenilins) mouse embryonic fibroblasts (MEFs), seeded on glass coverslips, were co-transfected with cDNAs coding for the ER-targeted Ca^{2+}-probe Aequorin (ER-Aeq) and PS2-T122R or the vector alone (pcDNA3); twenty-four to forty-eight hours after transfection, cellular Ca^{2+} stores were depleted by incubating the coverslips in a Ca^{2+}-free EGTA (600 μM) medium plus the SERCA inhibitor cyclopiazonic acid (CPA, 20 μM) to allow proper ER-Aeq reconstitution, and subsequently the steady state $[Ca^{2+}]_{ER}$ was analyzed by monitoring ER refilling upon addition of $CaCl_2$ (1 mM) to the bathing medium (see Fig.1 and M&Ms).

In all the cell lines tested, PS2-T122R expression significantly reduces $[Ca^{2+}]_{ER}$, (see Table 1).

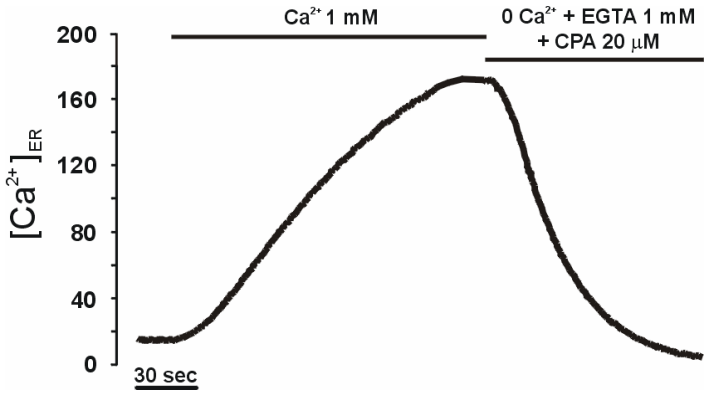

Fig.1: Representative trace of an ER-Aeq experiment. Briefly, cells reconstituted by incubation in a Ca^{2+}-free solution with EGTA 600 μM, CPA 20 μM and Coelenterazine N were initially bathed with a Ca^{2+}-free medium; ER refilling was monitored upon addition of Ca^{2+} ($CaCl_2$ 1 mM) to the external buffer, until a pleateau is reached (steady state $[Ca^{2+}]_{ER}$); subsequently, the medium was substituted with a Ca^{2+}-free, EGTA 1 mM and CPA 20 μM solution to re-empty the store (see text and M&Ms for details).

Table 1: Steady state ER Ca^{2+} levels (μM)

Cell type	Control	n	PS2-T122R	N	% Change
HeLa	414,4 ± 12,8	54	336,1 ±11,5 *	30	-19
SH-SY5Y	298,2 ± 9,6	28	140,5 ±8,0 ***	29	-53
wt MEFs	310,7 ± 27,0	27	180,0 ± 21,8 ***	9	-42
DKO MEFs	306,5 ±27,4	11	211,6 ± 16,1 ***	10	-31

Full length PS2 is necessary for reducing $[Ca^{2+}]_{ER}$

PS2 cell overexpression is reported to lead a marked increase in full length (FL) PS2, the immature form, and to a saturable increase in its dimeric, mature form, composed by N- and C-terminal fragments (NTF and CTF), because PSs endoproteolysis requires the formation of the γ-secrestase complex together with the other components (Aph-1, NCT and Pen-2, see "Introduction").

In order to investigate the molecular structure of PS2 mediating the reduction in $[Ca^{2+}]_{ER,}$ different PS2 constructs were expressed in a PSs null background (MEF DKO), together with ER-Aeq. The constructs used were: PS2 wt, the FAD-mutant PS2-T122R, the loss-of-function PS2-D366A, lacking endoproteolytic activity and thus remaining all in the FL form, a bicistronic construct coding simultaneously for both the PS2-NTF and CTF (PS2 NTF+CTF) resulting in mature PS2 (Stromberg et al., 2005), and finally the vector alone, as control. Western blot analysis revealed that, as expected, PS2 wt and T122R expression led to the production of both the FL and dimeric PS2 (detected as PS2-NTF as in fig. 2A or as PS2-CTF, data not shown), while only FL PS2 or dimeric PS2 were detected for PS2-D366A and PS2 NTF+CTF, respectively (Fig. 2A).

When cells were tested for $[Ca^{2+}]_{ER}$, only the three PS2 constructs leading to the formation of FL PS2 gave a reduction in $[Ca^{2+}]_{ER}$, while the expression of the dimeric construct left ER Ca^{2+} unchanged compared to control cells (Fig. 2B). In order to clarify whether a correlation between the effect on Ca^{2+} content and γ-secretase activity exists, the enzymatic activity was analyzed upon expression of the different constructs in PS null background DKO MEFs. It has been previously reported that the expression of the very same bicistronic construct in in

DB8 cells KO for both PSs reconstitutes γ-secretase complex and its activity (Strömberg et al., 2005). We confirmed this results in DKO MEFs by employing a recently developed γ-secretase cell assay (Florean et al. 2008). Cells were co-transfected with different PS2 constructs and one codifying the C99-GFP fusion protein, a substrate of γ-secretase; for each transfection, untreated cells and cells treated with γ-secretase inhibitor DAPT (1 μM), as internal negative control, were collected and analyzed by western blotting. Fig. 2C shows AICD-GFP fragment (the fusion peptide resulting from γ-secretase cleavage of C99-GFP) in untreated (-) or DAPT-treated cells (+): reconstitution of γ-secretase activity can be estimated by comparing the intensity of the AICD-GFP band in (-) and (+) lanes: PS2 WT and PS2 NTF+CTF expression gave a good reconstitution of γ-secretase activity, while PS2-T122R resulted in a small recovery, in agreement with the loss-of-function of this FAD-mutation, and PS2-D366A was completely devoid of proteolytical acivity.

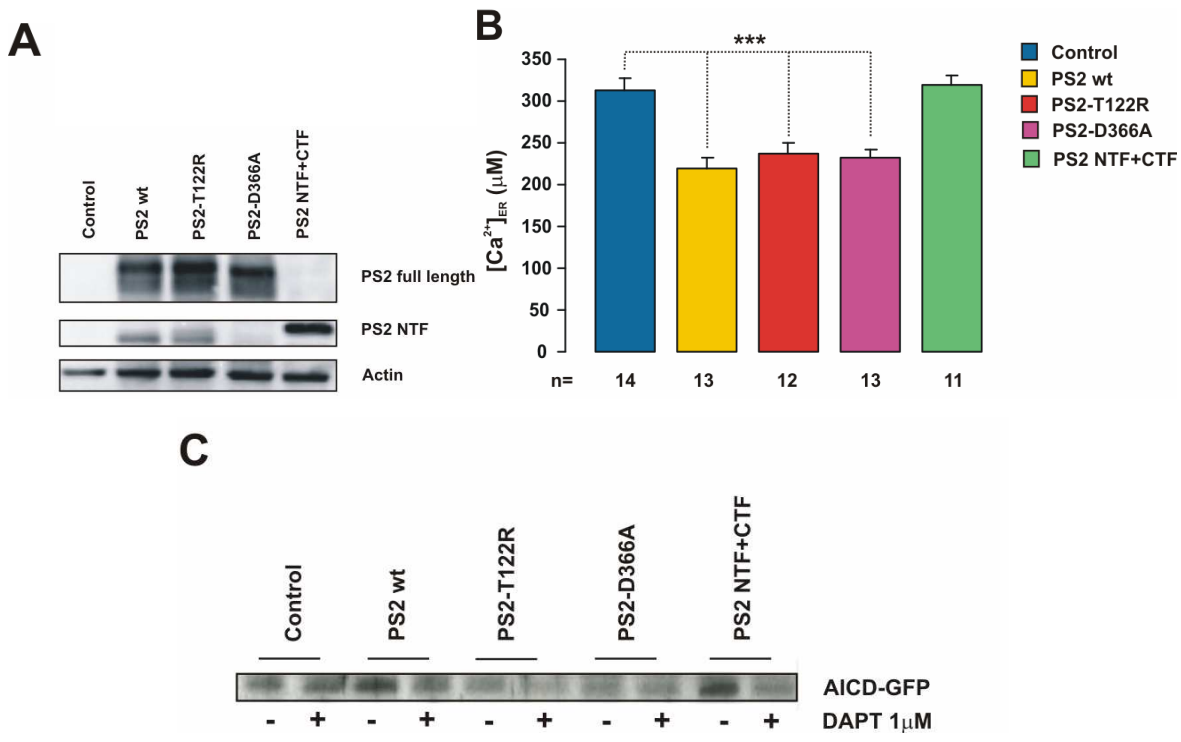

Fig.2: Effect of PS2 structure on $[Ca^{2+}]_{ER}$; 2A):expression levels (PS2 full length or PS2 NTF) of PS2 wt, PS2-T122R, PS2-D366A and PS2 NTF+CTF; PS2 NTF in the fifth lane migrates at higher MW because of a myc-tag; 2B) $[Ca^{2+}]_{ER}$ in cells transfected with the different PS2 constructs; 2C) γ-secretase assay based on AICD-GFP production in cell transfected with the different PS2 constructs and C99-GFP (see text for details).

The fact that NTF and CTF co-expression failed to mimic the effect of wt or mutant PS2 on Ca^{2+} handling despite it was able to reconstitute γ-secretase activity suggests that this latter is not required while the full length structure of PS2 is necessary for reducing $[Ca^{2+}]_{ER}$.
This idea could be further confirmed if a shift in the endogenous PS2 structure (mostly in the dimeric form) towards the FL form would result in a decrease in $[Ca^{2+}]_{ER}$.

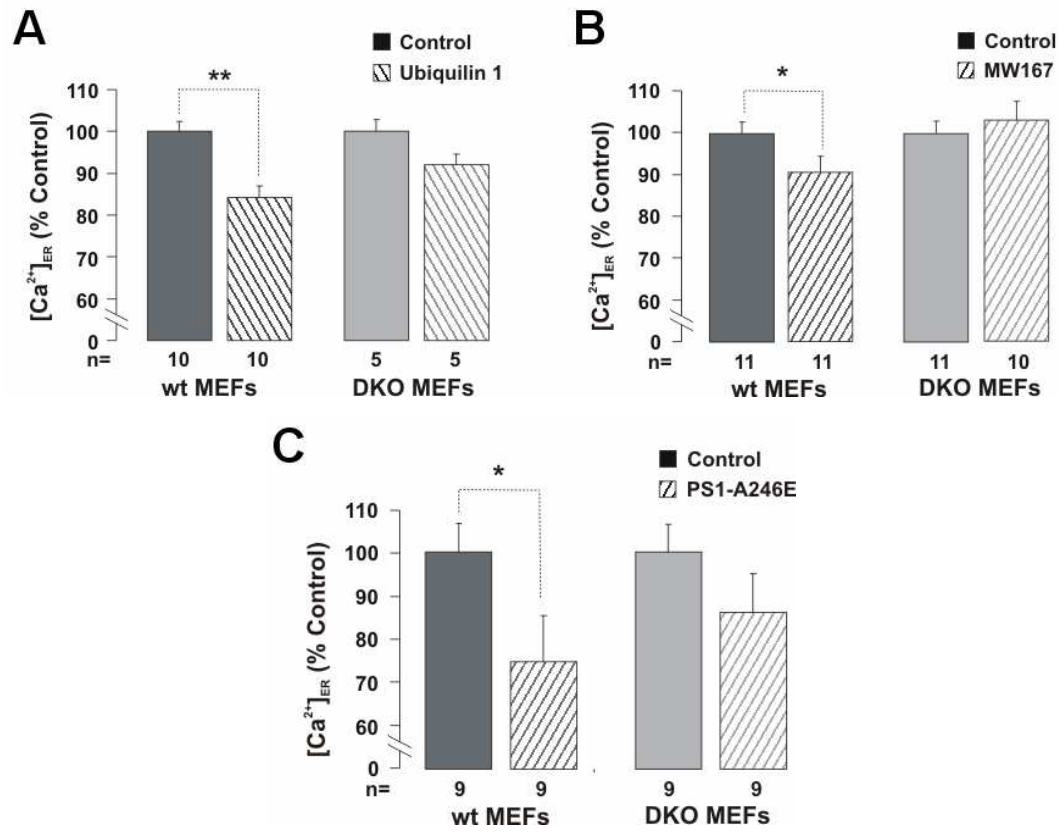

Fig.3: Increasing endogenous FL PS2 decreases $[Ca^{2+}]_{ER}$: 3A) Ubiquilin 1 expression protects PSs against maturation and reduces $[Ca^{2+}]_{ER}$ only in cells with endogenous PSs; 3B) overnight treatment with the presenilinase inhibitor MW167 decreases ER Ca^{2+} content only in PSs expressing cells; 3C) PS1-A246E overexpression affects $[Ca^{2+}]_{ER}$ only in presence of endogenous PS(2) (see text for details). Please note the axis interruptions.

Three different approaches have been applied for this purpose: (i) expression of the protein Ubiquilin 1 is reported to protect PSs from maturation (Massey et al., 2005); (ii) the pharmacological inhibitor MW167 (15 μM, overnight treatment) was shown to inhibit presenilinase activity, i.e. PS cleavage (Campbell et al., 2003) and, finally, (iii) PS1 can compete with PS2 for γ-secretase co-factors and thus saturate them preventing PS2 incorporation and maturation. For each approach, a comparison between MEF wt (with endogenous PS2 and PS1) and DKO MEFs (negative control, to exclude PSs independent

effects) transfected with ER-Aeq was performed. As can be seen in Fig. 3, in all the three cases only in MEF wt a significant decrease in $[Ca^{2+}]_{ER}$ was observed upon treatments, confirming that an increase in FL PS2 leads to a reduction in ER Ca^{2+} content. Moreover, the latter approach indicates that the FAD-mutant PS1-A246E does not reduces significantly $[Ca^{2+}]_{ER}$ in PS-null background.

An increased Ca^{2+} leak across ER membrane contributes to PS2-T122R effect

A decrease in $[Ca^{2+}]_{ER}$ can be essentially due to a deficit in the filling system (e.g. the SERCA activity) or to an increase in Ca^{2+} leakage across the ER membrane.

ER Ca^{2+} leak was evaluated in SH-SY5Y cells expressing ER-Aeq and PS2-T122R (or vector alone) measuring the rate of the decrease in $[Ca^{2+}]_{ER}$. After Aeq reconstitution (see M&Ms), cells were left to refill ER Ca^{2+} content till the steady state was reached; then, the $CaCl_2$-containing medium was substituted with a Ca^{2+}-free, EGTA (1 mM) and CPA (20 μM) containing solution, to visualize Ca^{2+} leak from the organelle (fig 4A).

Since the decay rate depends on the instantaneous $[Ca^{2+}]_{ER}$ and the ER steady state Ca^{2+} level is markedly different between control and PS2-T122R-overexpressing cells, we bathed the cells with media at different $[Ca^{2+}]$ (from 125 μM to 1 mM), allowing them to start from a wider range of ER steady state levels, and we followed the leak from different $[Ca^{2+}]_{ER}$ in both control and PS2-T122R cells; the single traces were aligned to CPA addition, as shown in Fig. 4B. For a clear comparison of the leakage in control and PS2-T122R cells, the average rate of Ca^{2+} leakage ($-d[Ca^{2+}]_{ER}/dt$) can be plotted on the instantaneous $[Ca^{2+}]_{ER}$ (fig 4C). As highlighted by the linear fits of the single points, the decay rate is higher (for $[Ca^{2+}]_{ER}$ above 40 μM) in PS2-T122R than in control cells (slope = 0,040 ± 0,003 s^{-1} and 0,032 ± 0,003 s^{-1} respectively, n= 24, $p<0,001$). On average, the slope in PS2-T122R expressing cells is increased by the 17% compared to the control cells (n=7).

When similar experiments were carried out in HeLa cells (Fig. 4D–F), the estimated slopes were: (s^{-1}) 0.031 ± 0.3, 0.035 ± 0.001, 0.031 ± 0.004 and 0.028 ± 0.003 for cells transfected, respectively, with the cDNA coding for PS2-T122R, PS2-M239I, PS2 wt and the void vector.

Collectively, these data suggest that an increased Ca^{2+} leak across the ER membrane is involved in the reduction of $[Ca^{2+}]_{ER}$ induced by PS2 expression.

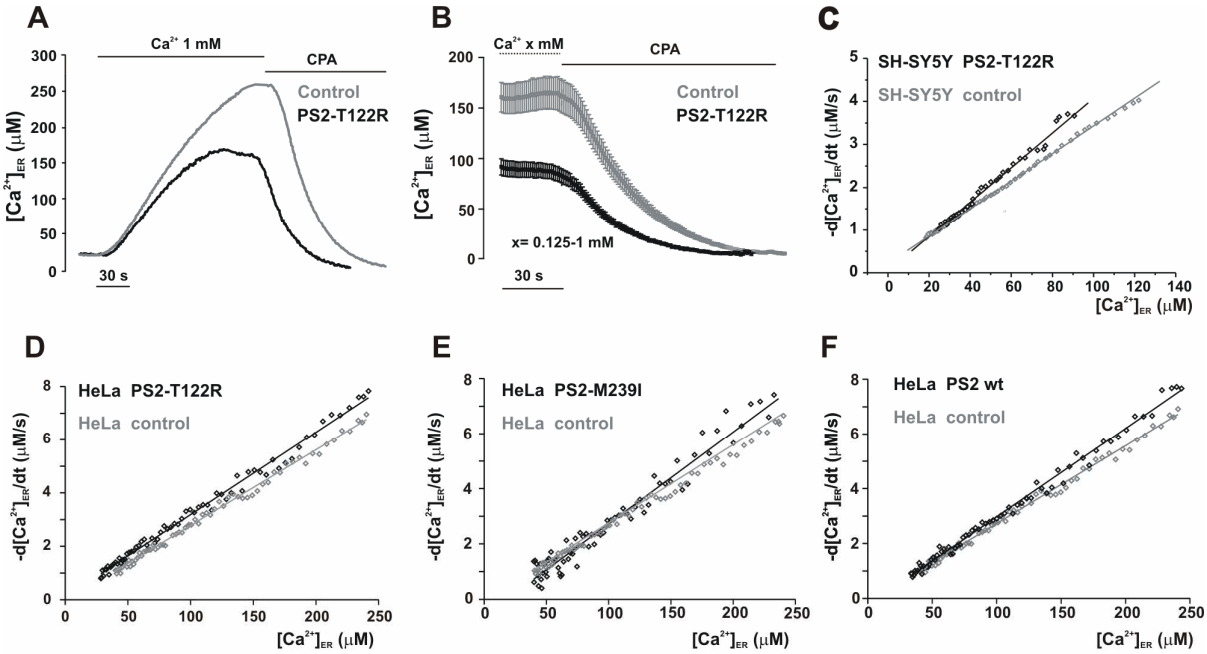

Fig. 4: Effects of PS2 on ER Ca^{2+} leak: 4A) representative traces of ER-Aeq experiments in cells expressing PS2-T122R (black) or void vector (grey); ER filling was obtained upon bathing the cells with Ca^{2+} 1 mM solution and, after a plateau is reached, Ca^{2+} leak was monitored by removing external Ca^{2+} and perfusing cells with 0 Ca^{2+}, EGTA 1 mM and CPA 20 μM solution; 4B) traces obtained bathing the cells with different [Ca^{2+}] (from 125 μM to 1 mM) are aligned to CPA addition; 4C) average rate of Ca^{2+} decay (from traces in Fig. 4B) plotted on instantaneous $[Ca^{2+}]_{ER}$ of PS2-T122R (black) and control (grey) SH-SY5Y cells; Fig.4D-F: average rate of Ca^{2+} decay plotted on instantaneous $[Ca^{2+}]_{ER}$ for Hela cells transfected with cDNA coding for PS2-T122R (Fig.3D, black), PS2-M239I (Fig.3E, black) and PS2 wt (Fig.3F, black) or void vector (grey).

Leak pathways potentially involved: RyRs, IP_3Rs and RTC

Two Ca^{2+} releasing channels present at the level of the ER membrane that can contribute to an increased leak of Ca^{2+} from the ER are RyRs and IP_3Rs.

In order to investigate the possible involvement of RyRs in PS2-T122R effect, a pharmacological approach was chosen: overnight incubation with the classical RyR inhibitor dantrolene (20 μM) caused only a minor but significant recovery (+12 %, $p<0,05$, Fig. 5B) of the PS2-dependent ER Ca^{2+} content reduction, as estimated by the cytosolic Ca^{2+} release upon addition of the IP_3-generating agonist Bradikin (Bk, 100 nM) and CPA (20 μM) in cells transfected with the cDNAs coding for PS2-T122R (or void vector) and cyt-Aeq (Fig. 5A) (see M&Ms for cyt-Aeq reconstitution and experimental protocol).

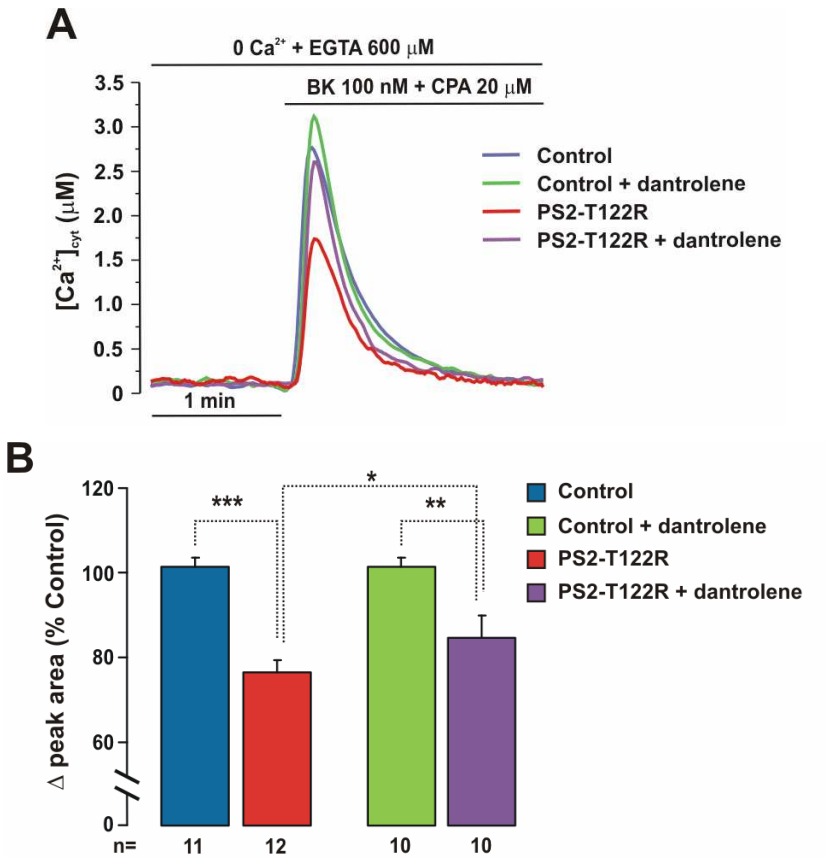

Fig 5: Involvement of RyRs in PS2-T122R effect on $[Ca^{2+}]_{ER}$; 5A) representative traces of cyt-Aeq experiments on PS2-T122R and control cells with or without overnight treatment with RyR's inhibitor dantrolene; 5B) averages of Ca^{2+} release (estimated as the area under the peak and expressed as a percentage of control) for each condition. Please note the axis interruption in the histogram.

The IP_3R contribution to the PS2-T122R effect on ER $[Ca^{2+}]$ was firstly investigated by a protocol of cell permeabilization that allows the washout of endogenous IP_3 that can activate sparse IP_3Rs even at cell resting condition. Cells transfected with ER-Aeq and PS2-T122R or void vector were first treated with digitonin (20-100 μM) and then perfused with an intracellular medium at constant free $[Ca^{2+}]$ (buffered at a constant level by an EGTA-based buffer; see M&Ms).

In DKO MEFs, the reduction in $[Ca^{2+}]_{ER}$, obtained upon expression of PS2-T122R, remains significant also in permeabilized cells (Fig. 6, full colour bars). In the same experimental conditions, the application of the IP_3Rs antagonist Heparin (100-200 μM), to exclude any possible activation of IP_3Rs, gave no recovery of the PS2-T122R effect (Fig. 6 striped bars).

Similar experiments were performed also on SH-SY5Y cells and the reduction in the steady-state ER Ca^{2+} level upon PS2-T122R expression was maintained (reductions in PS2-T122R expressing cells compared to control cells were 40 ± 1.4% and 41 ± 6%, mean ± sem, n=5, respectively with and without heparin).

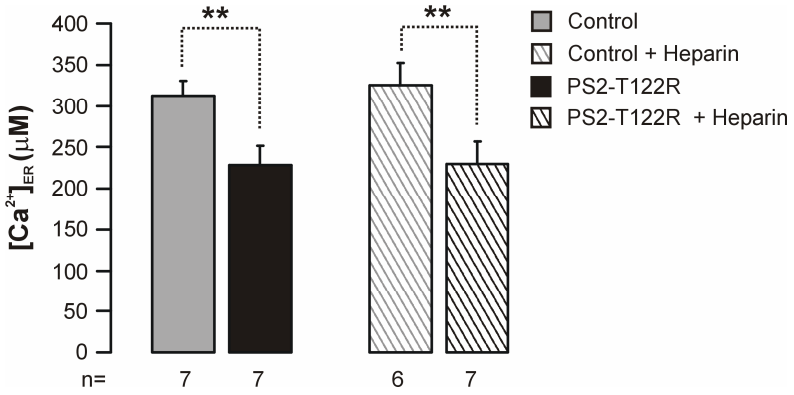

Fig. 6: PS2-T122R effect on $[Ca^{2+}]_{ER}$ is still significant in permeabilized cells and after IP_3Rs antagonist Heparin (100-200 μM) application (see text for details).

The second followed approach to evaluate the contribution of specific IP_3Rs isoforms to the PS2-T122R effect on ER Ca^{2+} content was their down-regulation by siRNAs.

MEFs are reported to express IP_3R-3 isoform at high levels in addition to IP_3R-1 (Kasri et al, 2006). wt MEFs were thus transfected with the cDNAs coding for ER-Aeq and PS2-T122R (or vector alone) and siRNAs specific for IP_3R-1, IP_3R-3 (Fig. 7) or both (data not shown): when $[Ca^{2+}]_{ER}$ was examined by ER-Aeq experiments, only the knock-down of IP_3R-3 gave a small but significant recovery (+ 10 %, $p<0,01$, Fig. 7A) of the PS2-T122R effect, while the knock-down of IP_3R-1 was ineffective (Fig. 7B). Protein down-regulation was confirmed by western blot analysis (Fig.7).

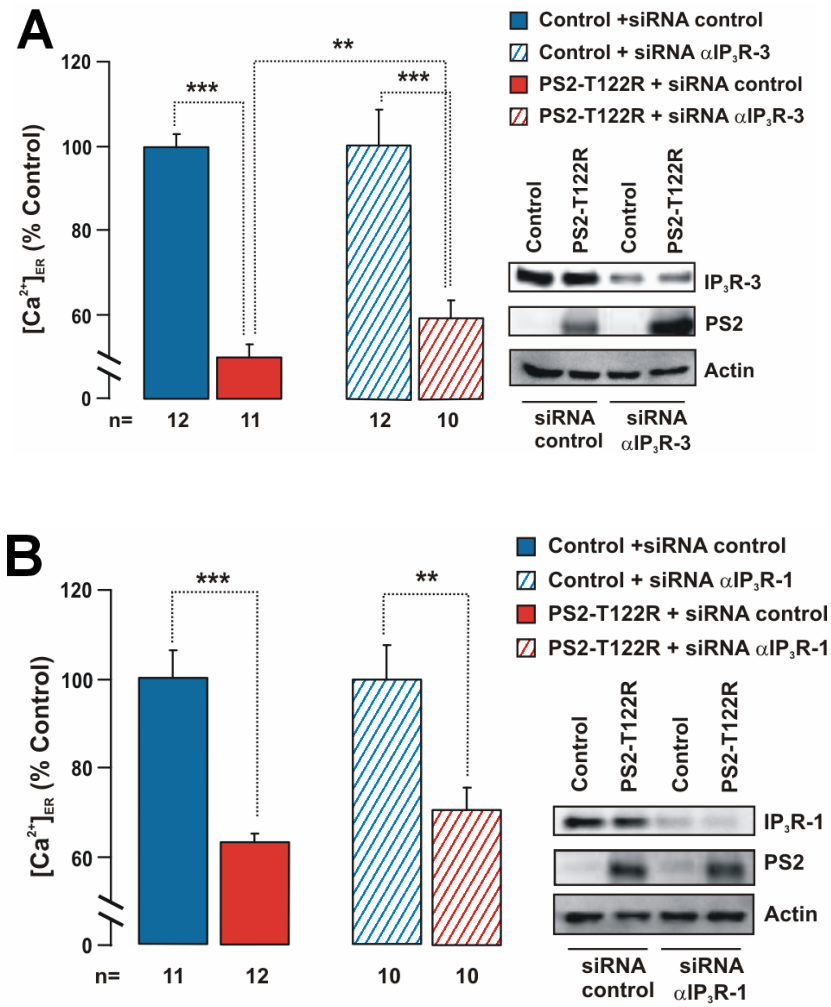

Fig. 7: Selective IP_3Rs isoforms down-regulation effect on PS2-T122R-induced $[Ca^{2+}]_{ER}$ reduction; 7A) the knock-down of IP_3R-3 (confirmed by western blotting) resulted in a small but significant recovery of $[Ca^{2+}]_{ER}$; 7B) the knock-down of IP_3R-1 (confirmed by western blotting) gave no significant recovery of $[Ca^{2+}]_{ER}$. Please note the axis interruption.

A further possible Ca^{2+} leak pathway in the ER is the Ribosomal-Translocon-Complex (RTC). This protein import machinery can allow the flux of Ca^{2+} ions once freed of the newly synthesized protein. Its possible implication in the PS2-T122R reduction of $[Ca^{2+}]_{ER}$ was investigated by using two different pharmacological agents: Puromycin (200 µM, preincubated for 10 minutes at 37°C before experiments) that should leave the RTC in an open configuration favouring Ca^{2+} leak, and the peptidyl-transferase inhibitor Anisomycin (200 µM, preincubated 1 hour at 4°C) that should lock the RTC in a closed configuration. As shown in Fig. 8, in SH-SY5Y cells over-expressing PS2-T122R $[Ca^{2+}]_{ER}$ remained significantly lower compared to control cells upon both treatments.

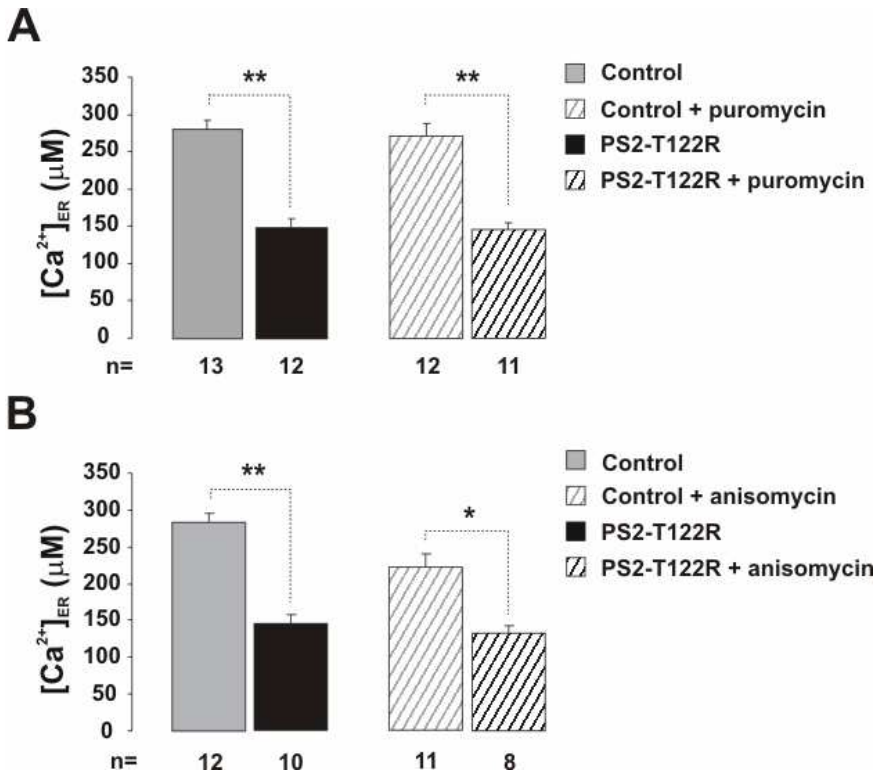

Fig. 8: Involvement of RTC in PS2-T122R effect on $[Ca^{2+}]_{ER}$: 8A) in SH-SY5Y cells preincubated with puromycin $[Ca^{2+}]_{ER}$ reduction due to PS2-T122R was unaffected; 8B) anisomycin treatment did not caused a significant recovery of PS2-T122R effect. (see text for details).

Altogether these data indicate that an increased Ca^{2+} leak across the ER membrane is partially involved in the reduction of $[Ca^{2+}]_{ER}$ due to PS2-T122R expression, although it is clearly not the major mechanism accounting for it. More specifically, RyRs and IP_3R(-3), but not RTC, seem to be involved in reducing $[Ca^{2+}]_{ER}$.

These results, together with other parallel studies conducted in the lab on PS2 effect on SERCA activity, were published in March 2009 (Brunello et al., 2009, see attached paper).

PS2 over-expression and mitochondrial Ca^{2+} uptake

In order to go further in the characterization of PS2 effect on Ca^{2+} homeostasis we analyzed whether and how this protein, and its FAD-linked mutations, affects mitochondrial Ca^{2+} handling. As described in the introduction (see "Introduction"), mitochondria are central

organelles in cellular Ca^{2+} homeostasis, and Ca^{2+} shuttling between ER and mitochondria strongly influences their activity.

Two additional points increase the interest in investigating ER-mitochondria interaction in FAD-PS2 expressing cells: firstly, IP_3R-3, suggested above as involved in PS2 effect, is supposed to be the IP_3R isoform more closely coupled with mitochondria (Mendes et al., 2005); secondly, while this part of my work (presented hereafter) was in progress, a paper showed that PSs are enriched in MAMs, ER membrane tightly associated with mitochondria (Area-Gomez et al., 2009).

As previously described, mitochondria uptake cytosolic Ca^{2+} that can enters the cell by PM channels or can be released from the intracellular stores, mainly the ER, by different Ca^{2+} channels. The latter case was investigated to understand whether PS2-T122R induces an alteration in ER-mitochondria coupling. A comparison between cytosolic and mitochondria Ca^{2+} responses can be done looking at Fig. 10: normally, when SH-SY5Y cells, bathed with a Ca^{2+}-free EGTA (600 μM) solution, are stimulated with the IP_3 generating agonist Bradikinin (Bk, 100 nM) and CPA (20 μM) Ca^{2+} is completely released from ER resulting in a prompt rise in $[Ca^{2+}]_{cyt}$ (with a peak around 2 μM, black); in the same conditions, mitochondria can transiently uptake Ca^{2+} above 100 μM (red).

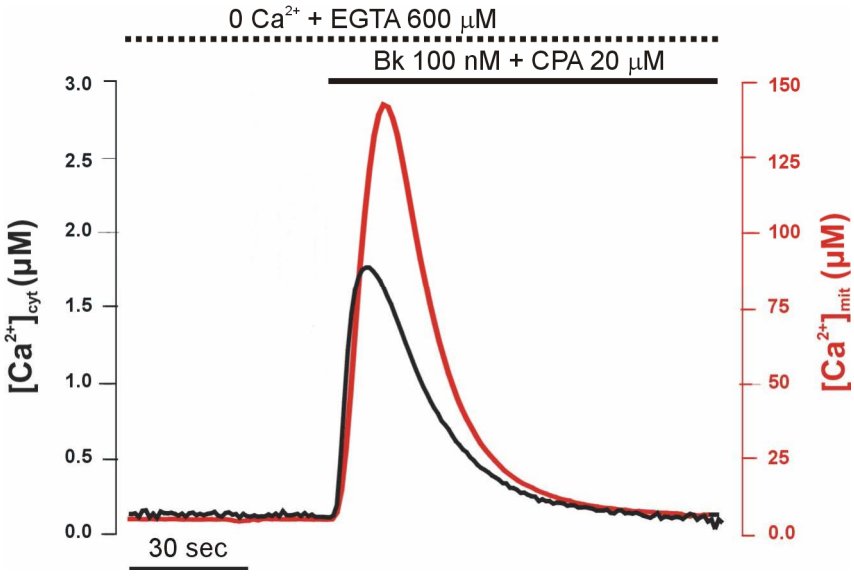

Fig. 10: Representative traces of cytosolic and mitochondrial Ca^{2+} responses in SH-SY5Y cells: cells were perfused in a Ca^{2+}-free medium with EGTA 600 μM and stimulated with Bradikinin (Bk) 100 nM and CPA 20 μM; this stimulation results in a cytosolic Ca^{2+} peak at around 2 μM (black) and a corresponding mitochondrial Ca^{2+} peak around 150 μM (red).

Mitochondria do not amplify $[Ca^{2+}]_{cyt}$ reduction in PS2-overexpressing cells

Mitochondria are supposed to amplify a difference in cytosolic Ca^{2+} release between two cell samples because their Ca^{2+} uptake mechanism relies on sensing high $[Ca^{2+}]$ cellular microdomains and on a low-affinity Ca^{2+} uniporter. Thus, since cells over-expressing PS2 (wt and T122R, see Fig.2B) have a reduced $[Ca^{2+}]_{ER}$ and consequently lower cytosolic Ca^{2+} rises upon stimulation (Fig. 11A,C), in the same cells mitochondria were expected to have dramatically reduced Ca^{2+} uptake. Actually, this was not the case: albeit reflecting the reduced cytosolic Ca^{2+} peaks, mitochondrial Ca^{2+} responses, in SH-SY5Y cells transfected with mitmut-Aeq and over-expressing PS2, do not show an amplified reduction in the face of the cytosolic one, compared with control cells (Fig. 11B,D).

To overcome differences in mitochondrial Ca^{2+} responses due to the various $[Ca^{2+}]_{ER}$, intracellular Ca^{2+} stores in void vector-transfected cells were partially emptied by incubating the coverslips in a Ca^{2+}-free EGTA (600 μM) solution, at 4°C for an appropriate time (usually between 15-45 minutes), to lower their $[Ca^{2+}]_{ER}$ to levels comparable to those present in PS2-overexpressing cells, as confirmed by cyt-Aeq measurements for each experimental set (Fig. 11A,C).

When compared with pre-emptied control cells ("control 0 Ca^{2+}/4°C"), PS2 wt and PS2-T122R expressing cells indeed showed higher mitochondrial peaks upon stimulation (Fig. 11B,D). In percentage, the increase in mitochondrial Ca^{2+} peaks was above 40% for PS2 wt expressing cells, and above 50 % for PS2-T122R expressing cells (Fig. 11E).

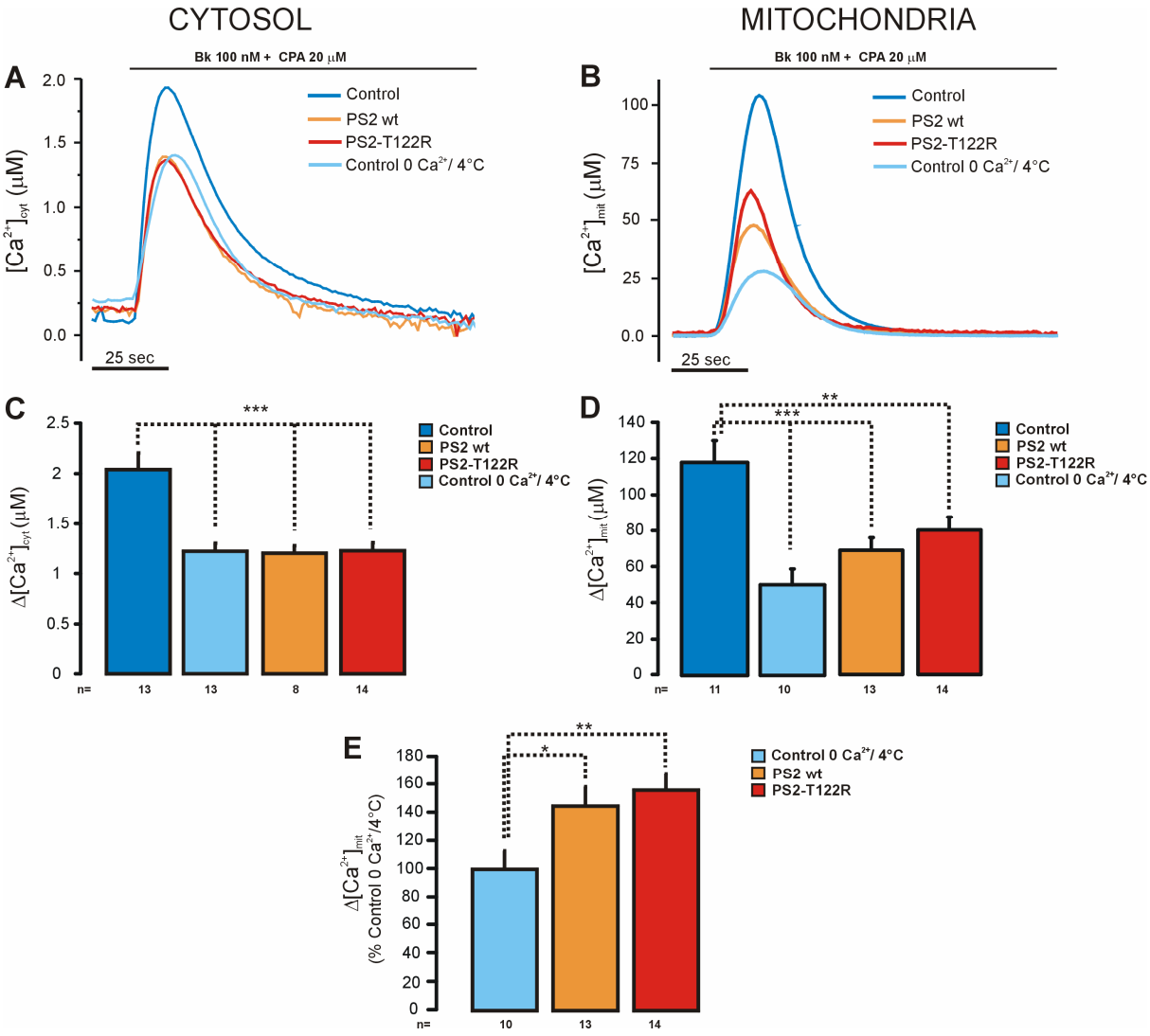

Fig.11: Comparison between cytosolic and mitochondrial Ca^{2+} peak in control, PS2 wt and PS2-T122R expressing cells: 11A: representative traces of cytosolic experiments; "control 0 Ca^{2+}/4°C" refers to control cells incubated in Ca^{2+}-free EGTA 600 μM solution to bring their $[Ca^{2+}]_{ER}$ to levels similar to those in PS2-transfected cells; cells were treated as previously described (Fig. 10); 11B) representative traces of the corresponding mitochondrial Ca^{2+} responses; 11C) bars representing the average values of cytosolic peaks (measured as delta between peak value and basal level, "$\Delta[Ca^{2+}]_{cyt}$") obtained for the different conditions in cytosolic experiments, note that control 0 Ca^{2+}/4°C, PS2 wt and PS2-T122R cells have the same $\Delta[Ca^{2+}]_{cyt}$; 11D) bars representing the average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$), note that PS2 wt and PS2-T122R expressing cells have higher mitochondrial peaks compared to pre-emptied control cells; 11E) bars representing the average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$) expressed as a percentage of pre-emptied controls.

PS2-overexpression does not directly affect mitochondrial Ca^{2+} uptake

The first possible explanation behind the increased mitochondrial Ca^{2+} uptake in PS2-expressing cells is a direct effect of PS2 on mitochondrial uptake efficiency.

In order to investigate whether mitochondrial Ca^{2+} uptake kinetics is increased in PS2-overexpressing cells, mitochondrial aequorin experiments were performed in digitonin permeabilized SH-SY5Y cells (see M&Ms) bathed with an intracellular medium with the free $[Ca^{2+}]$ buffered at a constant levels (1 μM, 10 μM and 30 μM) by an EGTA-based buffer. In this condition, mitochondria are exposed to an uniform $[Ca^{2+}]$ and thus mitochondria Ca^{2+} uptake depends exclusively on their uptake mechanisms.

In this condition, mitochondrial peaks in control, PS2-wt and PS2-T122R expressing cells are mostly comparable (only at 30 μM $[Ca^{2+}]$ PS2-T122R expressing cells show slightly increased mitochondrial peaks), as shown in Fig. 12.

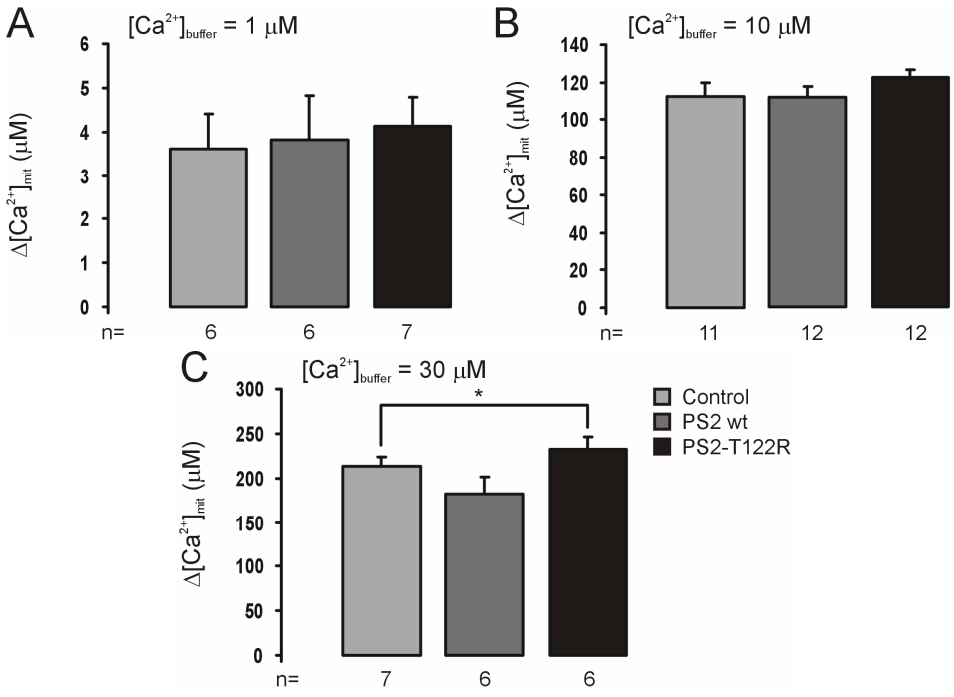

Fig. 12: Mitochondrial Ca^{2+} uptake in permeabilized cells; 12A) bars representing average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$) of cells bathed with an intracellular 1 μM $[Ca^{2+}]$ buffer; 12B) bars representing average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$) of cells bathed with an intracellular 10 μM $[Ca^{2+}]$ buffer; 12C) bars representing average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$) of cells bathed with an intracellular 30 μM $[Ca^{2+}]$ buffer.

PS2(-T122R) over-expression increases ER-mitochondria interaction

Another possible explanation for an increased mitochondrial Ca^{2+} uptake in PS2-expressing cells is a closer apposition between mitochondria and ER that would bring mitochondria at shorter distance from Ca^{2+} releasing sites on ER and thus allow them to sense higher [Ca^{2+}] micro-domains.

The interaction between mitochondria and ER was investigated by co-transfecting SH-SY5Y cells with PS2 wt or PS2-T122R (or void vector) together with a mitochondrial-targeted Red Fluorescent Protein (mt-RFP) and an ER-targeted GFP (ER-GFP), in parallel with standard transfection with aequorin probes for Ca^{2+} experiments. Cells expressing mitochondrial and ER fluorescent proteins were fixed and mounted with mowiol and subsequently analyzed by confocal microscopy (see M&Ms).

In transfected cells (transfection efficiency was above 70%) mitochondria were visualized as red, while ER was marked in green; when these two structures were close enough the two (red and green) signals overlapped, giving a yellow signal; actually, PS2-T122R expressing cells (Fig. 13C) showed a higher degree of signal overlapping and consequently of close ER-mitochondria proximity, if compared with control cells (Fig. 13A), while PS2 wt expressing cells (Fig. 13B) had a less evident increase in yellow signal. When a statistical analysis was performed on several cells from different transfections, ER-mitochondria interaction in PS2 wt expressing cells was modestly increased (+14.1 %), while PS2-T122R expressing cells had a robust increase in co-localization (+ 47.6 %), compared to control cells (Fig. 13D).

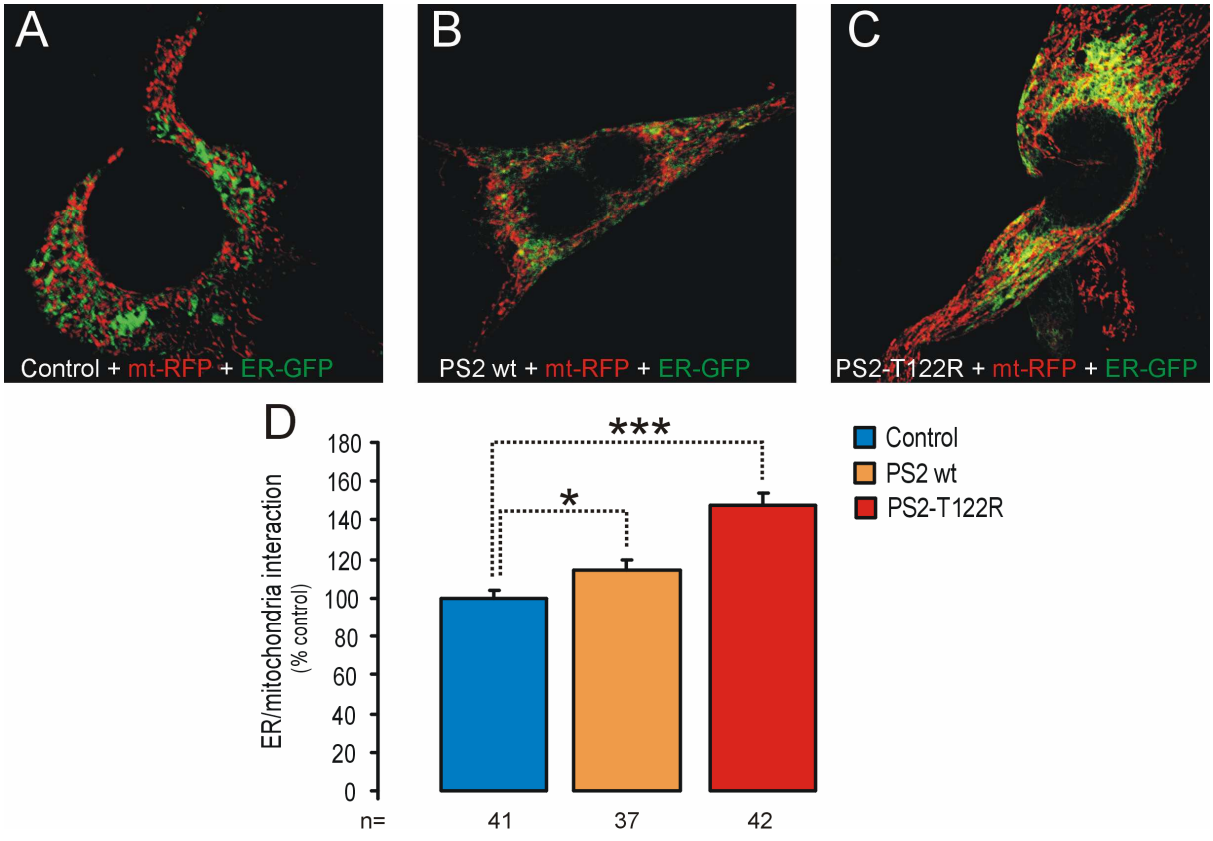

Fig. 13: ER-mitochondria interaction analyzed by confocal microscope analysis of control (13A), PS2 wt (13B) and PS2-T122R (13C) cells expressing mitochondrial-RFP (mt-RFP, red) and ER-GFP (green); yellow area indicate overlapping of the two signals and thus proximity of the two organelles; 13D) statistic analysis of ER-mitochondria interaction estimated as red/green overlapping surface on cell total surface, expressed as a percentage of control; PS2 wt and PS2-T122R over-expressing cells have a slightly (PS2 wt) or robustly (PS2-T122R) increased interaction.

These data suggested that PS2 can somehow modulate ER-mitochondria interaction and that the FAD-mutation is more potent than the wt form in coupling the two organelles.

Endogenous PS2 regulates ER-mitochondria interaction

Since the previous experiments were based on transient over-expression of the protein, it would be interesting to understand if also endogenous PS2 had a role in controlling the juxtaposition of ER and mitochondria.

siRNAs against PS2 (or control siRNA, 20 nM, see M&Ms) were thus used to down-regulate the endogenous protein level in SH-SY5Y cells transfected i) with cyt-Aeq and

mitmut-Aeq, for Ca^{2+} measurements or ii) with mt-RFP and ER-GFP for confocal microscope analysis. Protein down-regulation was confirmed by western blotting (Fig 14C).

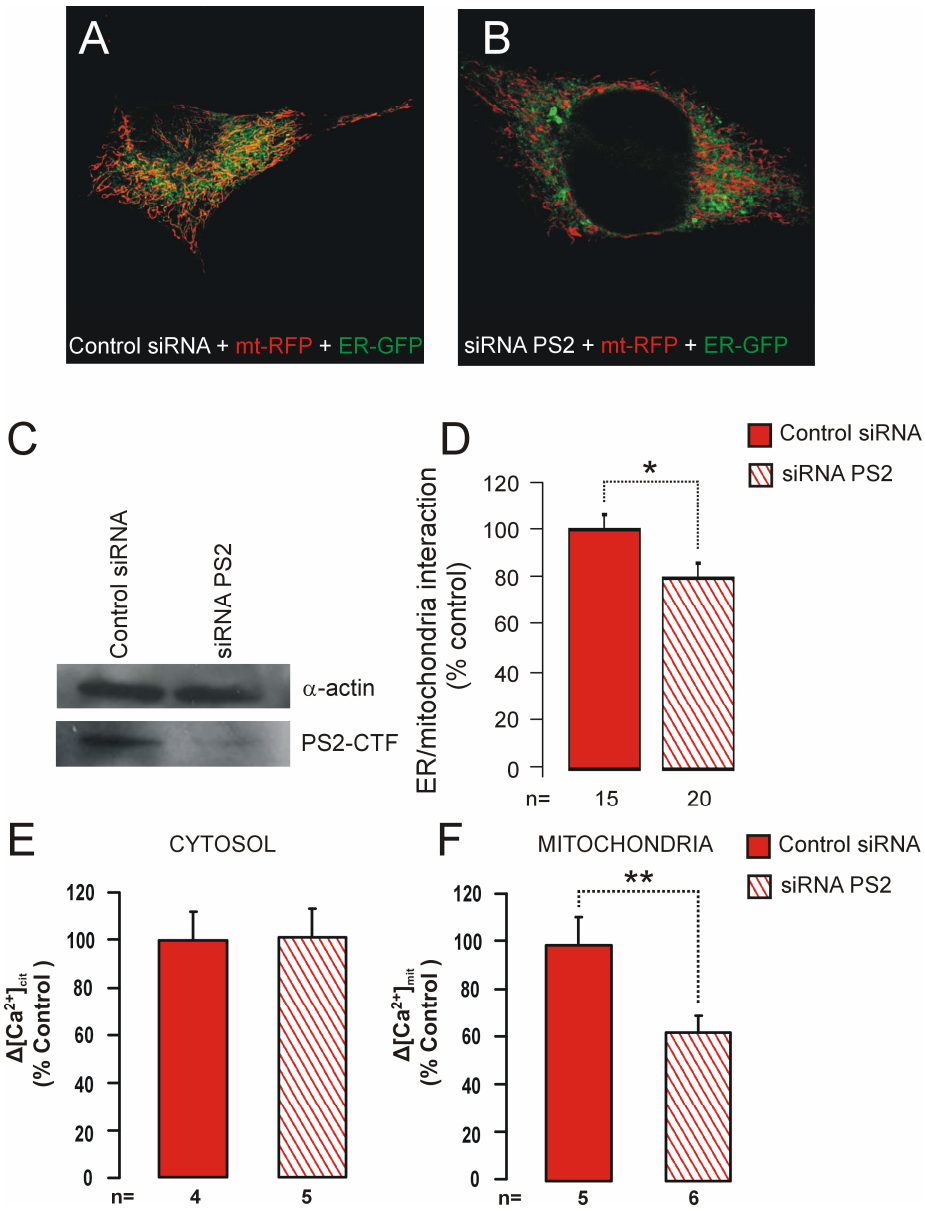

Fig. 14: Role of endogenous PS2 on ER-mitochondria interaction: 14A,B) confocal images of SH-SY5Y cells transfected with mt-RFP and ER-GFP and treated with control siRNA (14A) or PS2 siRNA (14B); 14C) western blotting showing down-regulation of PS2 in PS2 siRNA treated cells compared to controls; actin is showed as loading control; 14D) statistic analysis of ER/mitochondria interactions in siRNA-treated cells: PS2 down-regulation reduces the interaction; 14E) bars representing average values of cytosolic peaks ($\Delta[Ca^{2+}]_{cyt}$) expressed as percentage of control, showing no difference between the two treatments; 14F) bars representing average values of mitochondrial peaks ($\Delta[Ca^{2+}]_{mit}$) expressed as percentage of control: PS2 down-regulation reduces mitochondrial Ca^{2+} peaks even if cytosolic Ca^{2+} release is unchanged.

Aequorin experiments, performed as described above (stimulation with Bk 100 nM + CPA 20 μM in Ca^{2+}-free EGTA 600 μM extracellular buffer), showed that cytosolic Ca^{2+} release was comparable in the PS2 siRNA or control siRNA treated cells (Fig. 14E), while mitochondrial peaks were significantly reduced in PS2 knock-down cells (Fig. 14F).

Indeed, by confocal microscopy, a significant decrease in ER-mitochondria interactions was found (Fig. 14D) in PS2 siRNA treated cells (Fig.14B), compared to controls (Fig. 14A).

These data suggest that endogenous PS2 participates in controlling ER-mitochondrial apposition.

Single cells analysis with "Cameleon" probes of PS2-T122R effects on cytosolic and mitochondrial Ca^{2+} handling

Finally, a single cells analysis using FRET-based "Cameleon" probes (see "Introduction") was applied to SH-SY5Y cells transfected with PS2-T122R (or vector alone) and the cytosolic D3cpv (Fig 15C) or the mitochondrial 4mt-D1cpv (Fig. 15E) probes (see M&Ms for technical details).

As for aeqouorin experiments, cell were stimulated with Bradikinin (Bk, 100 nM) and CPA (20 μM), to discharge Ca^{2+} stores: the normalized difference between the ratio YFP/CFP peak, reached upon stimulation, and the basal fluorescence ratio (ΔR/R0, see M&Ms) was taken as a measurement of Ca^{2+} release, or Ca^{2+} uptake, in cytosol (Fig.15A) or mitochondria (Fig. 15B) experiments, respectively. Also with this type of analysis, a strong reduction was found in the cytosolic peaks reached by PS2-T122R expressing cells compared to controls (Fig. 15D); this reduction was not amplified in mitochondrial responses (Fig. 15F), in agreement with the previuos observation done by the cyt-Aeq and mitmut-Aeq experiments (see Fig. 11).

Altogether these data suggest a novel role for PS2: controlling ER-mitochondria interactions, it can modulate mitochondrial Ca^{2+} uptake, a key feature in cell physiology. The mechanisms behind this effect are however still under investigation.

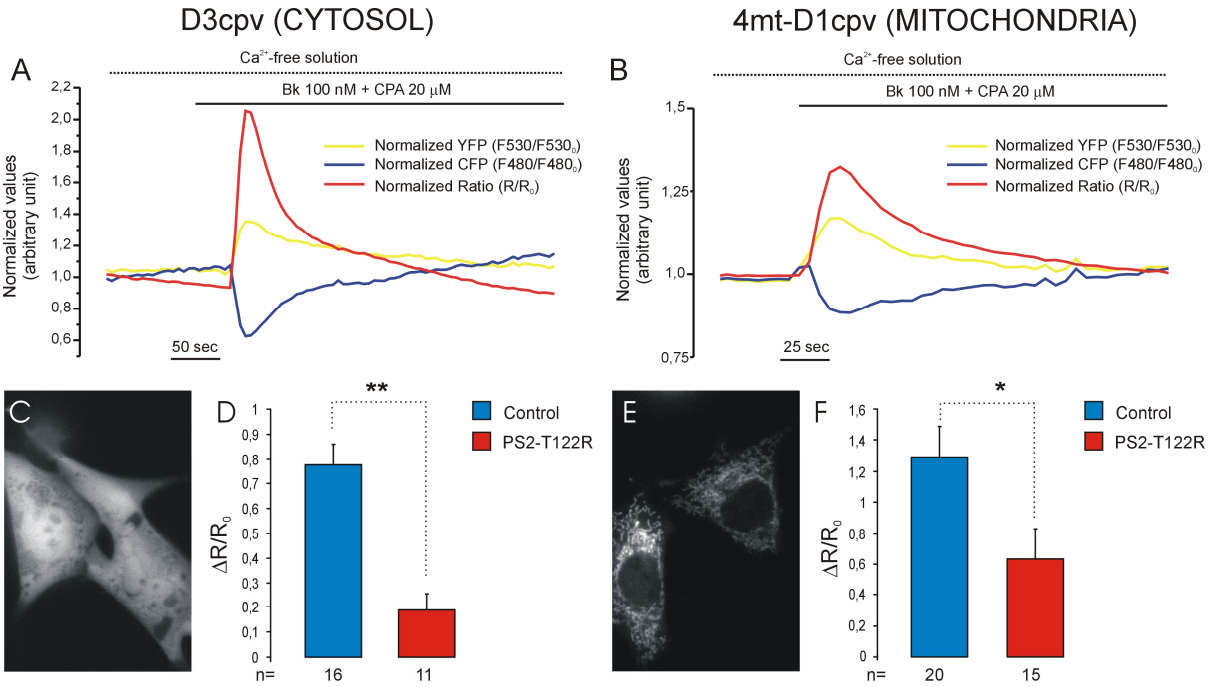

Fig 15: Single cells analysis of cytosolic and mitochondrial Ca^{2+} dynamics in cells over-expressing PS2-T122R; 15A,B) representative FRET traces of SH-SY5Y cells expressing the cytosolic D3cpv probe (15A) or the mitochondrial 4mt-D1cpv (15B) probe: yellow lines indicate YFP emission, while blue lines refer to CFP emission: note that they move in opposite directions when a variation in $[Ca^{2+}]$ occurs; red traces represent the YFP/CFP ratio; for a convenient presentation, normalized values are presented; 15C,E) fluorescence images (Λ= 480 nm) of cells expressing D3-cpv (15C) or 4mt-D1-cpv (15E); 15D,F): bars representing the average normalized $\Delta R/R0$ values of cytosolic peaks (15D) or mitochondrial peaks (15F) in void-vector transfected cells (blue) or PS2-T122R transfected cells (red).

DISCUSSION AND CONCLUSIONS

The data here presented stand for the evolution of previous works, carried out in the lab, showing a reduction of Ca^{2+} levels in the intracellular stores of cells expressing FAD-mutations in PS2 (and, at a lesser extent, also in PS1). The effect was found in skin fibroblast from FAD-patients, immortalized cell lines and primary rat cortical neurons transiently transfected with FAD-PSs (more prominently with FAD-PS2) and stable clones bearing PS2 FAD-mutants. Although very consistent among them, these data were still lacking a mechanistic explanation: why, and how, are Ca^{2+} stores reduced in the presence of FAD-PS(2)? The mechanisms behind PS2-T122R (the PS2 mutation most effective in reducing Ca^{2+} stores) -induced $[Ca^{2+}]_{ER}$ reduction have been taken under investigation, as well as the possible consequences of PS FAD-mutant expression on mitochondria Ca^{2+} handling.

Three main research lines have been followed: (i) the relationship between PS2 structure (immature, full-length or mature, dimeric) and its effect con $[Ca^{2+}]_{ER}$; (ii) the involvement of three already suggested possible ER Ca^{2+} leak pathways (e.g. the Ca^{2+}-releasing channels RyRs and IP_3Rs and the Ribosomal-Translocon Complex, RTC); (iii) the effect of PS2 expression on the interaction between ER and mitochondria, key organelles in Ca^{2+} homeostasis.

Firstly, the effect of PS2-T122R on several cell lines (HeLa and SH-SY5Y cells, wt and PS1/PS2 DKO Mouse Embryonic Fibroblasts, “MEFs”) was checked by transfecting the cells with ER-Aeq and PS2 (or its void vector) and directly estimating their $[Ca^{2+}]_{ER}$: in all cell lines, PS2-T122R invariably reduced ER Ca^{2+} content, albeit with different potency (e.g., the effect was more strong in SH-SY5Y cells, followed by wt MEFs, DKO MEFs and finally HeLa cells). Interestingly, in our hands wt and DKO MEF cells showed a very similar steady state ER Ca^{2+} level, arguing against previous reports stating that DKO MEFs have an increased (Tu et al., 2006) or a decreased (Kasri et al., 2006) $[Ca^{2+}]_{ER}$ compared to wt cells. These discrepancies are probably due to the fact that these cells, although coming from the same source, have evolved separately as different clones, with consequent adaptive changes that make quite difficult a direct link between the presence of the protein (PSs) and the Ca^{2+} responses. However, DKO MEFs are a precious tool to investigate the effect of specific PSs in a PS null background and, for this reason, they were chosen as an ideal cell model to study the role of PS2 structure on $[Ca^{2+}]_{ER}$. To this purpose, cDNAs coding for different PS2 were

transfected together with ER-Aeq: PS2 wt, PS2-T122R, a catalytically inactive PS2 (D366A), that is not cleaved to the mature form, a bicistronic construct expressing separately PS2 N-terminal and C-terminal fragments (PS2 NTF+CTF) or the vector alone, as control. The proper expression of these constructs was checked by western blotting analysis: PS2 wt and T122R resulted in the formation of both full length (FL) and cleaved PS2, while PS2-D366A gave rise only to the FL and PS2 NTF+CTF only to the separate fragments. Reconstitution of γ-secretase activity, upon different constructs expression, was also checked by a new cell assay based on AICD-GFP formation (see Florean et al, 2008): PS2 wt and PS2 NTF+CTF well restored enzyme activity, while the FAD mutation behaved as a partial loss of function protein; no secretase activity was found for the PS2-D366A mutant, as expected. Once examined for $[Ca^{2+}]_{ER}$, the three constructs linked to the generation of the FL PS2 (wt, T122R and D366A) were similarly effective in reducing $[Ca^{2+}]_{ER}$, while the bicistronic vector left it unchanged, compared to control cells. This result suggests that the FL PS2 is necessary to reduce $[Ca^{2+}]_{ER}$; moreover, the PS2 effect on Ca^{2+} stores is independent from γ-secretase activity, since the mutant PS2-D366A, avoid of both endoproteolysis and secretase activities, exerted the Ca^{2+} effect.

The experiments were however based on transient protein over-expression, a condition that lead to a massive increase in FL PS2; on the contrary, endogenous FL PS2 is barely detectable since most of the protein, once synthesized, quickly enters γ-secretase complex and undergoes maturation. It would be therefore interesting to test whether an increase in endogenous FL PS2 would cause a decrease in $[Ca^{2+}]_{ER}$. Ubiquilin-1 over-expression and MW167 inhibitor overnight treatment were used as tools to protect endogenous PS2 from maturation, as reported in the literature (Massey et al., 2005; Campbell et al., 2003); indeed both approaches leads to a reduction in $[Ca^{2+}]_{ER}$ in cells with endogenous PS, while the treatments were ineffective on DKO MEFs, as expected. As additional strategy, PS1 over-expression was carried out: transient cell over-expression of PS1 saturating all γ-secretase co-factors required for PS maturation, would statistically prevent PS2 maturation and increase its FL, immature level; actually, upon this experimental protocol, a decreased $[Ca^{2+}]_{ER}$ was found only in wt MEFs (with endogenous PS1 and PS2) but not in DKO MEFs. This is in agreement with the results of the two previous approaches, showing that the FL form of PS2 is necessary for reducing ER Ca^{2+} level. Moreover, this provides evidence that PS1 alone is not sufficient

to cause a reduction in $[Ca^{2+}]_{ER}$ and thus highlights the key role of PS2 in controlling Ca^{2+} homeostasis.

An increased Ca^{2+} leak across the ER membrane is one of the possible mechanisms for a $[Ca^{2+}]_{ER}$ reduction since ER steady state Ca^{2+} level depends on the balance between SERCA filling activity and the constitutive cations leak across the membrane. Assuming a constant SERCA activity, an increase in the leak would result in a decreased $[Ca^{2+}]_{ER}$.

ER Ca^{2+} leak was directly estimated by measuring the decay rate of $[Ca^{2+}]_{ER}$ in SH-SY5Y or HeLa cells transiently transfected with PS2 wt, PS2-T122R or other FAD-PS2 mutants and compared with control, void-vector-transfected cells. Cells over-expressing PS2 (wt as well as FAD-mutants) showed an increased Ca^{2+} leak across the ER membrane, e.g. 17% higher in PS2-T122R expressing cells compared to controls.

wt PSs have been reported to form a low conductance Ca^{2+} channel in the ER membrane that would account for the 80 % of total ER Ca^{2+} leak; however, FAD-mutations were reported to dramatically reduce the permeability of this channel, and thus the ER Ca^{2+} leak, resulting in an intracellular Ca^{2+} overload (Tu et al., 2006; Nelson et al., 2007). The data here presented show that PS(2) over-expression increases significantly, but not massively, ER Ca^{2+} leak and that this effect is shared by PS2 wt and FAD-linked PS2. Thus, an increased leak across ER membrane is involved in PS2-dependent reduction of $[Ca^{2+}]_{ER}$ and this effect is common to wt and FAD-PS2. The relatively small increase in Ca^{2+} leak upon PS2 over-expression rises some doubts about the channel-forming properties of PS2 itself and other molecular structures in the ER membrane could mediate this PS2-induced reduction in $[Ca^{2+}]_{ER}$.

By employing the pharmacological RyRs inhibitor Dantrolene, a small but significant recovery of PS2-T122R induced $[Ca^{2+}]_{ER}$ reduction was observed (+12%). A similar recovery was found by down-regulating, with specific siRNA, isoform 3 of the IP_3R (+ 10%), but not isoform 1, although IP_3 washout through cell permeabilization and IP_3R antagonist Heparin application were not sufficient to abolish PS2 Ca^{2+} effect. A third possible ER Ca^{2+} leak pathway was considered, the Ribosomal Translocon Complex (RTC): drugs that should keep the RTC in a closed (Anisomycin) or open (Puromycin) configuration had however no effect on PS2-induced $[Ca^{2+}]_{ER}$ reduction. It is interesting to notice that these two drugs poorly affected also controls' $[Ca^{2+}]_{ER}$, suggesting that in the cell model employed (SH-SY5Y cells)

the contribution of RTC to ER Ca^{2+} leak is minimal. Actually, anisomycin reduced $[Ca^{2+}]_{ER}$, an effect that is opposite of what one would have expected, considering that the drug should lock RTC in a closed configuration; probably this result was due to an aspecific effect that would require further analysis beyond the aim of this project.

Altogether these data suggest that the slightly increased Ca^{2+} leak across the ER membrane in PS2-over-expressing cells is mainly due to RyRs and IP_3Rs, while RTC is probably not involved. Increased activation of RyRs and IP_3Rs has already been reported to explain the exaggerated Ca^{2+} release observed in FAD-PSs expressing cell (Stutzmann et al., 2004 and 2006; Smith et al., 2005; Cheung et al., 2008). Again, however, our results argued against these conclusions, since no increase in cytosolic Ca^{2+} release has never been found and $[Ca^{2+}]_{ER}$ and cytosolic Ca^{2+} rise were invariably decreased in PS2, T122R as well as wt, expressing cells. The data here presented refer always to protein over-expression conditions that could somehow lead to an underestimation of any difference between PS2 wt and T122R; previous data from the lab, however, on stable clones as well as on fibroblasts from FAD patients, showed that FAD mutants are more effective than wt (endogenous) PS in reducing $[Ca^{2+}]_{ER}$ (Zatti et al., 2006 and 2004; Giacomello et al., 2005)

The observed increased Ca^{2+} leak across the ER membrane is clearly not sufficient to explain the dramatic reduction in $[Ca^{2+}]_{ER}$ caused by PS2 over-expression. Thus, another, and more powerful, mechanism might be considered. A decreased ER Ca^{2+} level can be due to an increase in Ca^{2+} leak or to an impairment in the filling mechanism, i.e. SERCA activity. Complementary studies carried out in the lab demonstrated that PS2(-T122R) strongly affects SERCA activity. Thus, it is possible to state that PS2 over-expression reduces $[Ca^{2+}]_{ER}$ by sligthly increasing Ca^{2+} leak across RyRs and $IP_3R(-3)$ and deeply impairing SERCA-dependent Ca^{2+} uptake (see Brunello et al., 2009).

A pathological meaning of this PS2-dependent reduction in $[Ca^{2+}]_{ER}$ can be finally hypothesized. An increase in intracellular Ca^{2+} content associated with an exaggerated Ca^{2+} signaling has been invoked by several groups to sustain an additional negative effect of PSs mutations in AD based on the fact that an excessive increase in cytosolic Ca^{2+} can be toxic for neurons and cells in general. Data in favour of this "Ca^{2+} overload" hypothesis were mostly based on FAD-PS1 mutations, while FAD-PS2 mutants are rare and less investigated. From the results of this work and from previous data of the lab, it resulted however clearly that (FAD)-PS2 decreases ER Ca^{2+} content while FAD-PS1 leaves its level unchanged (see PS1-

A246E effect on DKO MEFs; Fig.) or weakly decreased, depending on the cell model under investigation (Zatti et al., 2006). It is well known that FAD cases linked to PS1 mutations are not only the most common but also the most aggressive, with earlier onset and more severe phenotype if compared to those caused by FAD-PS2. Assuming that Ca^{2+} exerts a modulatory role on AD pathology – besides the other well documented AD pathological features, e.g. Aβ42 accumulation and synaptic loss - it is thus possible to speculate that PS2, inducing $[Ca^{2+}]_{ER}$ reduction, might be endowed of a lower toxicity, if compared to those in PS1. In fact, the differential effects on Ca^{2+} homeostasis can influence the neuronal (and glial) response to death stimuli, such as exposure to Aβ42 and reactive-oxygen species, and thus favoring or delaying disease onset and progression.

The final part of my work opens however new perspectives about the pathological implication of PS2-linked Ca^{2+} dysregulation, focusing on mitochondrial Ca^{2+} handling.

The experiments here presented demonstrate that PS2-T122R, and PS2 wt in a milder way, favors mitochondrial Ca^{2+} uptake by increasing the interactions between mitochondria and ER (but minimally affecting mitochondrial uptake mechanism), where Ca^{2+}-releasing channels involved in ER-mitochondria Ca^{2+} shuttling are located. The mechanisms behind this phenomenon are still under investigation. In particular, three possibilities should be listed.

Firstly, PS2 can physically link ER and mitochondria, acting as a tether; FAD-mutations (at least T122R) would be better (or longer-lasting) tethers than the wt form.

Secondly, a PS2-dependent γ-secretase substrate could contribute in coupling ER and mitochondria, and FAD-PS2, with impaired enzymatic activity, would be less efficient in cleaving it allowing a better interaction between the two organelles; in this latter case, however, it must be said that PS2 (or γ-secretase) would serve also as a docking site for the proper cell positioning of this linker-substrate, since endogenous PS2 down-regulation reduces ER-mitochondria coupling.

Thirdly, since mitochondrial movement are deeply influenced by Ca^{2+} ions (see "Introduction"), the increased ER-mitochondria interactions could be due also to the PS2-induced Ca^{2+} dysregulation: e.g., increased ER Ca^{2+} leak and/or impaired SERCA activity could generate an area with higher $[Ca^{2+}]$ at specific ER site, where mitochondria would be recruited by their Ca^{2+}-sensitive motor machineries.

All these three hypothesis will be taken under consideration and investigated, as well as the effect of PS1 in this phenomenon. As for $[Ca^{2+}]_{ER}$ reduction, this could be a PS2-selective effect, in agreement with the different specificities of the two homologue proteins.

A recent paper (Area-Gomez et al., 2009) demonstrating that PS1 and PS2 are enriched in MAMs – ER domains that closely interact with mitochondria – further enforces the idea that these two proteins have a key role in controlling ER-mitochondria interaction.

On the effect that this increased ER-mitochondria apposition could have in FAD pathology, it is worth to mention that ER-mitochondria cross-talk has multiple and potent effects in cell life. This especially because the Ca^{2+} exchange between the ER and mitochondria plays not only a critical role in regulating a variety of physiological processes, but also because it is involved in some aspects of cell death (Giacomello et al., 2007). For example, $[Ca^{2+}]$ within mitochondria it is known to activate three key metabolic enzymes located in the matrix with the overall result that an increase in mitochondrial Ca^{2+} uptake, stimulated by ER Ca^{2+} release, activates ATP production, matching aerobic metabolism to energy demand. In addition, considering that local $[Ca^{2+}]$ can have both a positive and a negative effect on the opening probability of the two best known ER Ca^{2+} release channels (the IP_3R and the RyR), mitochondria, by sequestering into their matrix part of the released Ca^{2+} may modify the cytosolic local Ca^{2+} level and thus the Ca^{2+} activation/inhibition of the ER channels themselves. On the other hand, a massive or/and a prolonged accumulation of Ca^{2+} into the mitochondria can lead to the opening of a large conductance pore in the inner mitochondrial membrane, swelling of the organelle, breakage of the outer membrane and release into the cytosol of a series of pro-apoptotic proteins.

Regard to PS2, two possibilities exist: does PS2(-T122R) favor mitochondrial Ca^{2+} uptake in a way that compensate the reduction in $[Ca^{2+}]_{ER}$ thus ensuring to mitochondria the proper Ca^{2+}signals to sufficiently stimulate their activity? Or does PS2(-T122R) cause excessive ER-mitochondrial coupling allowing toxic mitochondrial Ca^{2+}overload? The pathological implications deriving from these two alternative possibilities are quite distinct and imply a different role (positive or negative) for PS2 in AD genesis.

An over-expressing experimental system is however not suitable to answer these questions, since the big difference between $[Ca^{2+}]_{ER}$ observed in control and PS2-over-expressing cells would make difficult the comparison between their mitochondrial Ca^{2+} effects. On the other side, stable clones or FAD patients fibroblasts, in which Ca^{2+} alteration

closely reflects the pathological condition, would be better models to investigate the implication of this phenomenon.

Finally, as described in “Results” section, FRET-based probes provides excellent tools to study Ca^{2+}dinamics at the single cells level and to analyze the effect of PS2 on organelles Ca^{2+} handling. In particular, the possibility of expressing simultaneously probes targeted to different compartments would be extremely helpful for this study – e.g., the expression of a mitochondrial and of a nuclear (reflecting cytosolic Ca^{2+}changes) probes would allow to compare directly and in the same cells the relationship between cytosolic Ca^{2+} release and mitochondrial peak in control and PS2-T122R expressing cells.

Certainly the further investigation of FAD-PS2 induced alteration in ER-mitochondrial Ca^{2+}shuttling, together with the reduction in $[Ca^{2+}]_{ER}$, will cast new light on the role of Ca^{2+} alteration in AD.

MATERIALS AND METHODS

Cell lines and transfection

HeLa and SH-SY5Y cells were grown in DMEM supplemented with 10% FCS containing penicillin (100 U/ml) and streptomycin (100 µg/ml).

For aequorin experiments, cells were seeded on coverslips (13 mm diameter) and allowed to grow to 50% confluence. At this stage, transfections of HeLa cells were carried out using the Ca^{2+}-phosphate technique in the presence of 4 µg of DNA [3 µg PS2-cDNA or void vector plus 1 µg aequorin (Aeq) cDNA]. SH-SY5Y cells were transfected by means of Lipofectamine^{TM}2000 using 1.5 µg of DNA (1 µg PS2-cDNA or void vector plus 0.5 µg Aeq cDNA). Intracellular Ca^{2+} measurements were carried out 48 or 24 h after transfection by means of the Aeq technique as previously described (Brini et al., 1995) and summarised below. PS1/PS2-null (DKO) and wild-type mouse embryonic fibroblasts (MEFs), obtained as previously described (Nyabi et al., 2002; Herreman et al., 1999), were kindly provided by Dr. Bart De Strooper (Center for Human Genetics, KUL, VIB, Leuven, Belgium). Cells, grown in DMEM-F12 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin, were transfected by Lipofectamine^{TM}2000 employing 2 µg of DNA (1.5 µg PS2-cDNA or void vector plus 0.5 µg Aeq cDNA). For γ-secretase activity assay (Florean et al., 2008) cells were transfected with 1.5 µg of PS2-cDNA and 0.5 µg of C99-GFP instead of aequorin. For ER-mitochondria interaction analysis cells aequorin cDNA was substituted with the corresponding amount of ER-GFP together with mt-RFP (ratio 1:1). For Ubiquilin-1 overexpression experiments, wt and DKO cells were transfected with 1,5 µg of Ubiquilin-1 cDNA (or void vector) and 0,5 µg of Aequorin cDNA. For RNA interference experiments, the growth medium was substituted 1 hour before transfection with antibiotics-free medium; siRNAs (murine IP_3R-1 (GenBank accession no. NM-010585; nucleotides 505–523, 2254–2272, 3680–3698, and 5122–5140), and IP_3R-3 (GenBank accession no. NM-080553; nucleotides 1114–1132, 1125–1143, 1219–1237, and 1459–1477); PS2, target sequence: GAUAUACUCAUCUGCCAUG; siGENOME RISC-Free Control siRNA; Dharmacon Research, Lafayette, CO) were added to the transfection mixes to a final concentration of 20-40 nM.

For FRET experiments, cells were seeded onto glass coverslips (24-mm diameter) and transfection was performed at 50% confluence with Lipofectamine^{TM}2000 using 3 μg of DNA (2 μg PS2-cDNA or void vector plus 1 μg of "Cameleon" probe cDNA); FRET measurements have been usually performed 24 h after transfection.

Aequorin Ca^{2+} measurements

Cells seeded on 13 mm diameter coverslips and transfected with cyt-Aeq or mitmut-Aeq constructs were incubated at 37°C with coelenterazine (5 μM) for 1-2 h in a modified Krebs–Ringer buffer (mKRB, in mM:140 NaCl, 2.8 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 glucose, pH 7.4) and then transferred to the perfusion chamber. For reconstitution of ER-Aeq, luminal [Ca^{2+}] was reduced before coelenterazine addition by exposing the cells to CPA (20 μM) in mKRB without $CaCl_2$ (Ca^{2+}-free mKRB) and containing EGTA (600 μM). Upon 1h incubation at 4°C in the same medium, the cells were extensively washed with Ca^{2+}-free mKRB supplemented with EGTA (1 mM) and bovine serum albumin (BSA, 2%). All the luminescence measurements were carried out in mKRB at 37°C. For SH-SY5Y and MEF cells, a high potassium medium (in mM: KCl 100, NaCl 43, $MgCl_2$ 1, HEPES 10, pH 7.4) was used. The experiments were terminated by cell permeabilization with digitonin (100 μM) in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to discharge the remaining unused Aeq pool. The light signal was collected as previously described (Brini et al., 1995).

For permeabilization, cells were exposed for 1-2 minutes to digitonin (20-100 μM) in an intracellular medium containing (in mM): KCl 130, NaCl 10, KH_2PO_4 1, succinic acid 2, $MgSO_4$ 1, HEPES 20, EGTA 0.05 pH 7, at 37°C. .The cells were then washed with the same intracellular medium containing EGTA 50 μM for 2-5 minutes. The Ca^{2+}-buffer solution was prepared by adding to the intracellular medium: HEDTA, piruvic acid and $MgCl_2$ (1 mM each), EGTA or BAPTA (2 mM) and $CaCl_2$ at different concentrations (0.5-1.8 mM) and the pH was brought to 7 at 37°C. $ATPNa_2$ (0.2 mM) was added to this Ca^{2+}-buffered solution. The free [Ca^{2+}] (0,1-2 μM) was estimated by MaxChelator2.5 and checked by fluorimetric measurements with fura-2.

FRET Ca^{2+} measurements

Cells expressing the fluorescent probes were analyzed using an inverted fluorescence microscope (Leica DMI 6000CS, Leica Microsystem, Wetzlar, Germany) equipped with a filter wheel and an immersion oil objective (40 X, N.A. 1.25). Excitation light produced by a Hg (100 W) lamp (Leica, type 11307072071/BZ.00) was filtered at the appropriate wavelength (425 nm) through an excitation filter, and the emitted light was collected through a Beamsplitter (OES s.r.l., Padua) (emission filters HQ 480/40M (for CFP) and HQ 535/30M (for YFP)) and a dichroic mirror 515 DCXR. The beamsplitter permits the collection of the two emitted wavelengths at the same time, thus preventing any artefact due to movement of the organelles.

Images were acquired using a cooled OES camera (OES s.r.l., PD) attached to a 12-bit frame grabber. Synchronization of the filter wheel and OES camera was performed through a control unit ran by custom-made Roboscope software; this software was also used for image analysis. Exposure time and frequency of image capture varied from 100 ms to 200 ms and from 0.5 to 1 Hz, respectively, depending on the intensity of the fluorescent signal of the cells analyzed and on the speed of fluorescence changes.

Cells were mounted into an open-topped chamber and maintained in an extracellular medium (modified Krebs-Ringer Buffer (mKRB, see above). Cytosolic (D3cpv probe) and mitochondrial (4mtD1cpv probe) experiments are started in 1 mM $CaCl_2$; after addition of EGTA 2 mM, to chelate external Ca^{2+}, cells are stimulated by applying Bradikinin (Bk, 100 nM) and CPA (20 μM); thereafter, Ca^{2+} ionophore ionomycin is applied to completely discharge the stores and finally a saturating $CaCl_2$ concentration (18 mM) is added (plus TRIS-HCl 10 mM to avoid acidification) in order to verify the dynamic range of each probe.

Off-line analysis of FRET experiments was performed with ImageJ software (Wayne Rasband, Bethesda,USA). YFP and CFP images were subtracted of background signals and distinctly analyzed after selecting proper regions of interest (ROIs) on each cell; subsequently, a ratio between YFP and CFP emissions was calculated (R=F530/F480). Data are presented as a normalized ratio (R/R_0, instantaneous ratio R divided for starting ratio R_0).

Plasmids

pcDNA3 vectors, codifying different PS2 mutants (M239I, N141I, T122R, D366A) were created by site directed mutagenesis of pcDNA3/PS2-wt (QuikChange Site-directed mutagenesis Kit, Stratagene, La Jolla, CA). The constructs were checked by sequence analysis (ABI Prism Genetic Analyzer 310, Applied Biosystems, Monza, Italy).

Protein extracts preparation and Western blot analysis

The different cell types were harvested and treated as previously reported (Giacomello et al., 2005). Briefly, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested with RIPA buffer supplemented with proteases inhibitors cocktail (Complete Mini™, Roche). Samples were analyzed in SDS-PAGE gel and Western blotting immunodetection was carried out with the polyclonal antibody anti-PS2 (324-335; Ab-2, Calbiochem, Merck, Darmstadt, Germany) and with the monoclonal mouse antibody anti-PS2 (MMS-359S, Covance Research Products Inc.). IP_3R-1 and -3 were detected by a polyclonal antibody (PA3-901A, ABR-Affinity BioReagents, Inc.) and a monoclonal mouse antibody (610312, BD Biosciences Pharmingen), respectively. AICD-GFP was detected by the polyclonal anti-GFP antibody ab290, purchased from abCAM (Cambridge Science Park, UK). Actin was detected by the monoclonal mouse antibody (A4700, Sigma-Aldrich) The proteins were visualised by the chemiluminescence reagent ECL (Amersham, GE Healthcare, UK Ltd Amersham PlaceLittle Chalfont Buckinghamshire HP7 9NA England).

Confocal images acquisition and analysis

Cells were fixed in Phosphate Buffered Saline (PBS) containing 4% paraformaldehyde and 20% saccharose for 15 min, incubated with 50mM NH_4Cl for 20 min, washed wih PBS and mounted with Mowiol (Sigma-Aldrich Saint Louis, MI). Images were collected at Leica TCS SP2 (Leica Microsystem, Wetzlar, Germany) confocal system by using the Arg 488 nm laser line and He-Ne 543 nm laser line, with an immersion oil objective (63X, N.A. 0.9) . Video microscopy was performed at 1024×1024 pixels per image with Leica Confocal

Software, with a 200 image lines per second acquisition rate. Images were elaborated with ImageJ program.

Chemicals and reagents

Antibiotics, sera, culture media, plasmids and Lipofectamine^{TM}2000 were purchased from Invitrogen (Carlsbad, CA, USA); DAPT, MW167 (γ-secretase inhibitor II) and ionomycin were from Calbiochem (Merck KGaA; Darmstadt, Germany) while all other reagents were from Sigma Chemical Co. (St. Louis, Mo), unless otherwise stated.

Statistical analysis

Data were analyzed by Origin 7.5 SR5 (OriginLab Corporation). Averages are expressed as mean ± s.e.m. (n=number of independent experiments; ∗ = $p < 0.05$, ∗∗=$p<0.01$, ∗∗∗ = $p < 0.001$, unpaired Student's t test).

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Presenilin-2 dampens intracellular Ca^{2+} stores by increasing Ca^{2+} leakage and reducing Ca^{2+} uptake

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Abstract

We have previously shown that familial Alzheimer's disease mutants of presenilin-2 (PS2) and, to a lesser extent, of presenilin-1 (PS1) lower the Ca^{2+} concentration of intracellular stores. We here examined the mechanism by which wild-type and mutant PS2 affect store Ca^{2+} handling. By using HeLa, SH-SY5Y and MEFs as model cells, and recombinant aequorins as Ca^{2+} probes, we show evidence that transient expression of either wild-type or mutant PS2 increases the passive Ca^{2+} leakage: both ryanodine- and IP_3-receptors contribute to Ca^{2+} exit out of the ER, whereas the ribosome translocon complex is not involved. In SH-SY5Y cells and MEFs, wild-type and mutant PS2 potently reduce the uptake of Ca^{2+} inside the stores, an effect that can be counteracted by over-expression of SERCA-2B. On this line, in wild-type MEFs, lowering the endogenous level of PS2 by RNA interference, increases the Ca^{2+}-loading capability of intracellular stores. Furthermore, we show that in PS double knockout MEFs, reduction of Ca^{2+} stores is mimicked by the expression of PS2-D366A, a loss-of-function mutant, uncleaved because also devoid of presenilinase activity but not by co-expression of the two catalytic active fragments of PS2. In summary, both physiological and increased levels of wild-type and mutant PS2 reduce the Ca^{2+} uptake by intracellular stores. To exert this newly described function, PS2 needs to be in its full-length form, even if it can subsequently be cleaved.

Keywords: presenilin • calcium stores • Alzheimer's disease • SERCA • aequorin

Introduction

Alzheimer's disease (AD) is responsible for the most part of the dementias in developed countries. Although the majority of AD cases are sporadic, a significant fraction of AD is inherited in a dominant pattern. The familial forms of AD (FAD) have been traced to mutations in genes for three proteins: the amyloid precursor protein (APP), presenilin-1 and -2 (PS1 and 2). PSs are essential components of the γ-secretase complex which, in turn, by cleaving APP in concert with β-secretase, produces the neurotoxic β-amyloid peptide (Aβ).

Much attention has recently been devoted to the fact that some FAD-linked PS mutants cause a dysregulation of cellular Ca^{2+} homeostasis. Ca^{2+} is a key parameter in neuronal physiopathology, as it controls many cellular functions, whereas alterations in Ca^{2+} levels are responsible for neuronal cell death in a number of genetic and sporadic diseases. It has been suggested that an imbalance of Ca^{2+} homeostasis may represent an early event in the pathogenesis of FAD, but the mechanisms through which FAD-linked PS mutants affect the control of cellular Ca^{2+} are controversial. In particular, it was observed that mutations in PS1 cause larger Ca^{2+} release from intracellular stores and increase excitotoxicity in neurons from transgenic mice [1–5]. The idea that FAD-linked PS mutations are somehow correlated to altered Ca^{2+} signalling was further supported by the fact that these mutations could alter the expression, or sensitivity, of endoplasmic reticulum (ER) Ca^{2+} release channels, ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate receptors (IP_3Rs) in different cell models [2, 6–11] and in neurons from AD mice [5, 12, 13]. Interestingly, similar observations were also made in non-neuronal cells, such as fibroblasts, lymphocytes and oocytes, indicating that PSs play a general role in Ca^{2+} homeostasis [11, 14]. These data lead to the 'Ca^{2+} overload' hypothesis [15, 16], stating that AD neuronal degeneration depends on exaggerated ER Ca^{2+} release because of ER Ca^{2+} overload. However, an increased ER Ca^{2+} content has not always been observed: different studies have described either no alteration or reduced ER Ca^{2+} stores in cells expressing wild-type

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(wt) or FAD-mutant PSs [11, 17–19]. In particular, we demonstrated that the FAD-linked PS2 mutations M239I and T122R reduce rather than increase Ca^{2+} release in fibroblasts from FAD patients and in cell lines stably or transiently expressing the PS2 mutants [18, 20, 21]. In addition, an extended investigation of other FAD-linked PS mutants (PS2−N141I, PS1-A246E, PS1-L286V, PS1-M146L, PS1-P117L), by monitoring directly the ER Ca^{2+} levels in different cell lines, confirmed that these FAD-linked PS2 mutations caused a reduction in ER Ca^{2+} levels and none of the PS1 mutations caused an increase [18].

Recently, a physiological role for wt PSs in ER Ca^{2+} handling has also been proposed, although divergent and contrasting data were reported [11, 18, 22–25]. In agreement, different mechanisms of PSs action have been proposed to explain their effect on Ca^{2+} homeostasis. PSs were demonstrated to form Ca^{2+} permeable channels in planar lipid bilayers [23], with FAD-linked PS mutants forming channels with reduced ionic conductance and thus being responsible for ER Ca^{2+} overload [23, 26]. An increased Ca^{2+} content has been also explained by an increased sarco-endoplasmic reticulum calcium-ATPase (SERCA)-2B activity, as deduced by accelerated cytosolic Ca^{2+} clearance following expression of wt PS1/2 or PS1−M146V in *Xenopus laevis* oocytes [25]. In contrast, Cheung *et al.* demonstrated that different FAD PS mutants increase IP_3Rs' sensitivity, leading to exaggerated Ca^{2+} responses, yet in the presence of reduced ER Ca^{2+} levels [11].

By employing different cell models, we here show that wt and mutant PS2 act primarily by reducing SERCA pumps' activity and secondly by increasing the leak across ER Ca^{2+} channels (RyRs and IP_3Rs).

Materials and methods

Cell lines and transfection

HeLa and SH-SY5Y cells were grown in DMEM supplemented with 10% FCS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Before transfection, cells were seeded on cover slips (13-mm-diameter) and allowed to grow to 50% confluence. At this stage, transfections of HeLa cells were carried out using the Ca^{2+}-phosphate technique in the presence of 4 μg of DNA (3 μg PS2-cDNA or void vector plus 1 μg aequorin [Aeq] cDNA). SH−SY5Y cells were transfected by means of Lipofectamine™2000 using 1.5 μg of DNA (1 μg PS2-cDNA or void vector plus 0.5 μg Aeq cDNA). Intracellular Ca^{2+} measurements were carried out 48 or 24 hrs after transfection by means of the Aeq technique as previously described [27] and summarized below. PS1/PS2-null ($PS1^{-/-}$, $PS2^{-/-}$) and wild-type mouse embryonic fibroblasts (MEFs), obtained as previously described [28, 29], were kindly provided by Dr. Bart De Strooper (Center for Human Genetics, KUL, VIB, Leuven, Belgium). Cells, grown in DMEM-F12 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin, were transfected by Lipofectamine™2000 employing 2 μg of DNA (1.5 μg PS2-cDNA or void vector plus 0.5 μg Aeq cDNA). For γ-secretase activity assay [30] cells were transfected with 1.5 μg of PS2-cDNA and 0.5 μg of C99-GFP instead of Aeq. For RNA interference experiments, the growth medium was substituted 1 hr before transfection with antibiotics-free medium; siRNAs (murine IP_3R-1 [GenBank accession no. NM-010585; nucleotides 505–523, 2254–2272, 3680–3698 and 5122–5140] and IP_3R-3 [GenBank accession no. NM-080553; nucleotides 1114–1132, 1125–1143, 1219–1237 and 1459–1477]; mouse PS2, target sequence: GAUAUACUCAUCUGCCAUG; siGENOME RISC-Free Control siRNA; Dharmacon Research, Lafayette, CO) were added to the transfection mixes to a final concentration of 20–40 nM.

Ca^{2+} measurements

Cells seeded on 13-mm-diameter cover slips and transfected with Aeq constructs were incubated at 37°C with coelenterazine (5 μM) for 1–2 hrs in a modified Krebs–Ringer buffer (mKRB, in mM:140 NaCl, 2.8 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 glucose, pH 7.4) and then transferred to the perfusion chamber. For reconstitution of ER-Aeq, luminal [Ca^{2+}] was reduced before coelenterazine addition by exposing the cells to CPA (20 μM) in mKRB without $CaCl_2$ (Ca^{2+}-free mKRB) and containing EGTA (600 μM). Upon 1-hr incubation at 4°C in the same medium, the cells were extensively washed with Ca^{2+}-free mKRB supplemented with EGTA (1 mM) and bovine serum albumin (BSA, 2%). All the luminescence measurements were carried out in mKRB at 37°C. For SH-SY5Y and MEF cells, a high potassium medium (in mM: KCl 100, NaCl 43, $MgCl_2$ 1, HEPES 10, pH 7.4) was used. The experiments were terminated by cell permeabilization with digitonin (100 μM) in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to discharge the remaining unused Aeq pool. The light signal was collected as previously described [27].

For permeabilization, cells were exposed for 1–2 min. to digitonin (20–100 μM) in an intracellular medium containing (in mM): KCl 130, NaCl 10, KH_2PO_4 1, succinic acid 2, $MgSO_4$ 1, HEPES 20, EGTA 0.05 pH 7, at 37°C. The cells were then washed with the same intracellular medium containing EGTA 50 μM for 2–5 min. The Ca^{2+}-buffer solution was prepared by adding to the intracellular medium: HEDTA, piruvic acid and $MgCl_2$ (1 mM each), EGTA or BAPTA (2 mM) and $CaCl_2$ at different concentrations (0.5−1.8 mM) and the pH was brought to 7 at 37°C. $ATPNa_2$ (0.2 mM) was added to this Ca^{2+}-buffered solution. The free [Ca^{2+}] (0.1–2 μM) was estimated by MaxChelator2.5 and checked by fluorimetric measurements with fura-2.

Plasmids

pcDNA3 vectors, codifying different PS2 mutants (M239I, N141I, T122R, D366A) were created by site-directed mutagenesis of pcDNA3/PS2-wt (QuikChange Site-directed mutagenesis Kit, Stratagene, La Jolla, CA). The constructs were checked by sequence analysis (ABI Prism Genetic Analyzer 310, Applied Biosystems, Monza, Italy).

Protein extracts preparation and Western blot analysis

The different cell types were harvested and treated as previously reported [21]. Briefly, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested with RIPA buffer supplemented with proteases inhibitors cocktail (Complete Mini™, Roche). Samples were analysed in SDS−PAGE gel, and Western blotting immunodetection was carried out

with the polyclonal antibody anti-PS2 (324–335; Ab-2, Calbiochem, Merck, Darmstadt, Germany) and with the monoclonal mouse antibody anti-PS2 (MMS-359S, Covance Research Products Inc.). IP_3R-1 and -3 were detected by a polyclonal antibody (PA3–901A, ABR-Affinity BioReagents, Inc.) and a monoclonal mouse antibody (610312, BD Biosciences Pharmingen), respectively. SERCA-2B detection was carried out with a polyclonal anti-SERCA-2 antibody (N-19, Santa Cruz Biotechnology, Inc.). AICD-GFP was detected by the polyclonal anti-GFP antibody ab290, purchased from abCAM (Cambridge Science Park, UK). Actin was detected by the monoclonal mouse antibody (A4700, Sigma-Aldrich) The proteins were visualized by the chemiluminescence reagent ECL (Amersham, GE Healthcare, UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK).

Chemicals and reagents

Antibiotics, sera, culture media, plasmids and Lipofectamine^{TM}2000 were purchased from Invitrogen (Carlsbad, CA); DAPT and MW167 (γ-secretase inhibitor II) were from Calbiochem (Merck KGaA; Darmstadt, Germany), whereas all other reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Statistical analysis

Data were analysed by Origin 7.5 SR5 (OriginLab Corporation). Averages are expressed as mean ± S.E.M. (n = number of independent experiments; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, unpaired Student's t-test).

Results

Effect of PS2 variants on store calcium leak

Using cytosolic and organelle-targeted Aeq, we have previously demonstrated that expression of various PSs, especially the PS2 variants, reduces the steady-state free Ca^{2+} concentration of the endoplasmic reticulum ($[Ca^{2+}]_{ER}$) and Golgi apparatus ($[Ca^{2+}]_{GA}$), the major intracellular Ca^{2+} stores of mammalian cells [18]. For those experiments, we used primarily HeLa cells, a convenient and extensively used cell model. In the work here presented, we carried out the same type of measurements in two other cell types, the SH-SY5Y cell line, derived from a human neuroblastoma, and mouse embryonic fibroblasts (MEFs). The latter cell model offers the possibility that a PS double knockout (DKO) clone is also available [31].

SH-SY5Y cells were cotransfected with the cDNAs coding for a recombinant Aeq targeted to the endoplasmic reticulum (ER-Aeq) and for PS2-T122R, a FAD-linked mutant PS2 whose effect at the Ca^{2+} store level was originally described in human FAD fibroblasts and HeLa cells [18, 21]; control cells were transfected with ER-Aeq and vector alone (pcDNA3). Twenty-four to 48 hrs after transfection, Ca^{2+} stores were depleted in a Ca^{2+}-free, EGTA-containing medium to allow ER-Aeq reconstitution (see Materials and Methods) and subsequently the refilling process was continuously monitored upon addition of $CaCl_2$ (1 mM) to the bathing medium. Under these conditions, the $[Ca^{2+}]_{ER}$ increased in a couple of minutes up to a plateau that stabilized at a significant lower level in PS2−T122R–expressing cells, with respect to control cells (Fig. 1A). Table 1 reports the steady-state $[Ca^{2+}]_{ER}$ obtained with this protocol in all the cell types here investigated: HeLa, SH-SY5Y, wt and DKO MEFs. Note that PS2-T122R was maximally effective in SH-SY5Y cells (–53 ± 3%, mean ± S.E.M., $n = 29$).

The question then arises as to the molecular mechanisms leading to this reduced steady-state $[Ca^{2+}]_{ER}$. A first possibility is that it is due to an increased Ca^{2+} leak out of the stores. The passive ER Ca^{2+} leak rate was thus measured directly using a previously described procedure [32]. Briefly, cells, cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R (or the void vector), were first allowed to refill their emptied stores until they reached a steady-state. In the typical experiment with SH−SY5Y cells, shown in Fig. 1B, a wide range of external Ca^{2+} concentrations (0.125–0.25–0.5–1 mM) was used in order to obtain different levels of the steady-state $[Ca^{2+}]_{ER}$ before leak measurement; typically, three cover slips, for both control and mutant PS2–expressing cells, were used at each Ca^{2+} concentration. At the plateau, the SERCA inhibitor cyclopiazonic acid, CPA (20 μM), was added in a Ca^{2+}-free medium containing EGTA (1 mM) to remove extracellular Ca^{2+} and the rate of $[Ca^{2+}]_{ER}$ decay was continuously monitored (Fig. 1A and B). The single traces were averaged and aligned to CPA addition (grey and black traces for control and PS2-T122R–expressing cells, respectively). Figure 1C shows the average rate of Ca^{2+} leakage ($-d[Ca^{2+}]_{ER}/dt$) as a function of the instantaneous $[Ca^{2+}]_{ER}$, estimated from the traces shown in Fig. 1B (grey and black symbols, for control and PS2-T122R–expressing cells, respectively). In cells expressing the mutant PS2, at $[Ca^{2+}]_{ER}$ above 40 μM, the decay rates are significantly higher than those of controls. The linear fit of the experimental data shown in Fig. 1C thus shows a significantly greater slope for PS2-T122R–expressing cells compared with control ones, being respectively (s^{-1}) 0.040 ± 0.003 and 0.032 ± 0.002 ($n = 24$, $P < 0.001$). The percentage increase in slope, averaged among different cell batches, was 17.1 ± 6.3% ($n = 7$). When similar experiments were carried out in HeLa cells (Fig. 1D–F), the estimated slopes were (s^{-1}) 0.031 ± 0.3, 0.035 ± 0.001, 0.031 ± 0.004 and 0.028 ± 0.003 for cells transfected, respectively, with the cDNA coding for PS2-T122R, PS2-M239I, wt PS2 and the void vector. Compared with SH−SY5Y cells, expression of PS2-T122R in HeLa cells resulted in a smaller increase in slope (9.1 ± 2.3%, $n = 3$) (see Table 1 and [18]).

It has to be mentioned that in control, void-vector transfected SH-SY5Y and HeLa cells (Figs 1C and 2), the absolute values of the decay rates were rather similar for the same ER Ca^{2+} level, that is, around 2.8−3 μM/sec. at 100 μM $[Ca^{2+}]_{ER}$ for the two cell types. Indeed, in control cells, ER leak rates covered higher ranges in HeLa (0−12 μM/sec.) than in SH-SY5Y (0−6 μM/sec.) being the latter cell type characterized by a lower maximal steady-state ER Ca^{2+} level (see Table 1).

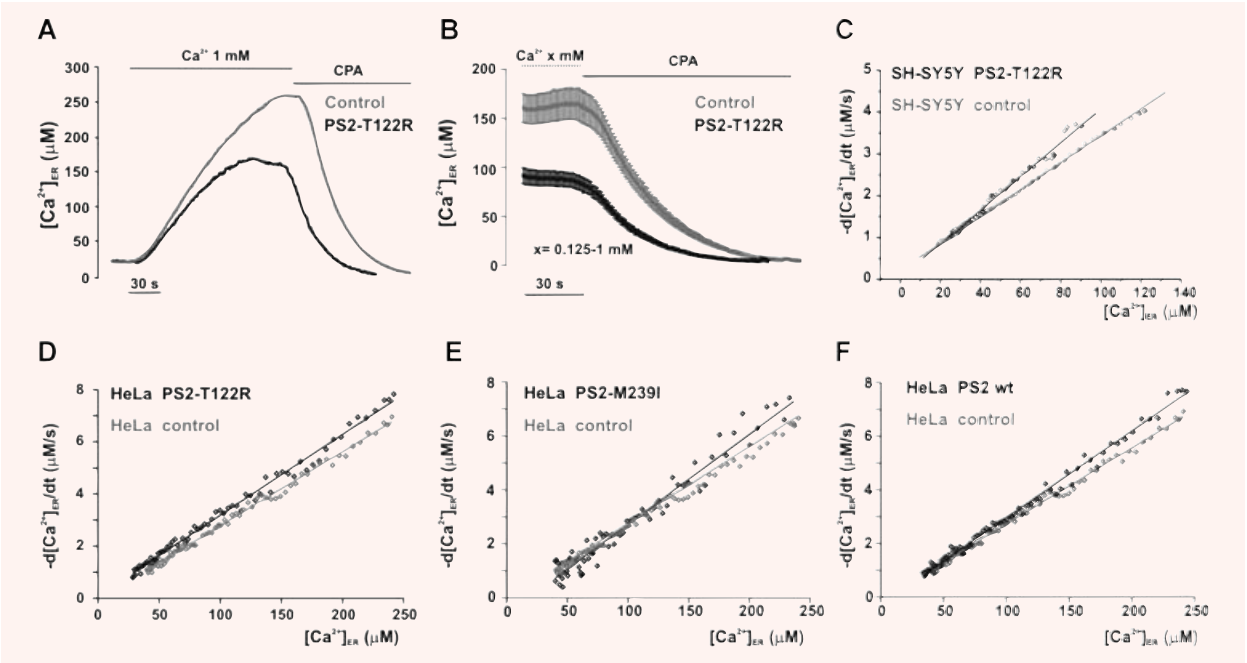

Fig. 1 Effect of PS2 variants on passive ER Ca^{2+} leak. (**A**) Representative traces of $[Ca^{2+}]_{ER}$ measurements in SH-SY5Y cells transiently cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R (black) or with the void vector as control (grey). After Aeq reconstitution (see Materials and Methods), cells were washed and bathed in a Ca^{2+}-free, EGTA (0.6 mM)-containing medium before exposure to $CaCl_2$ (1 mM). The passive ER Ca^{2+} leak was estimated by addition, at the plateau, of CPA (20 μM) together with EGTA (1 mM). (**B**) For quantitative analysis of ER Ca^{2+} leak, different steady-states of $[Ca^{2+}]_{ER}$ were obtained by the addition of $CaCl_2$ ranging from 0.125 to 1 mM. The single traces were averaged and aligned to CPA addition (grey and black traces for control and PS2-T122R–expressing cells, respectively, mean ± S.E.M., $n = 24$). (**C**) The rate of ER Ca^{2+} loss ($-d[Ca^{2+}]/dt$) was plotted as a function of the instantaneous $[Ca^{2+}]_{ER}$ estimated from single traces as shown in (**B**) (black and grey symbols, for PS2-T122R-expressing and control cells, respectively). The S.E.M. was omitted for clarity. (**D**, **E**) Experiments with HeLa cells were carried out as described in (**A**) and analysed as shown in (**B**) and (**C**) (black and grey symbols for PS2-expressing and control cells, respectively, $n = 6$–9).

Table 1 ER Ca^{2+} levels (μM)

Cell type	Control	*n*	PS2-T122R	*n*	% change
HeLa	414.4 ± 12.8	54	336.1 ± 11.5*	30	−19
SH-SY5Y	298.2 ± 9.6	28	140.5 ± 8.0***	29	−53
wt MEFs	310.7 ± 27.0	27	180.0 ± 21.8***	9	−42
DKO MEFs	306.5 ± 27.4	11	211.6 ± 16.1***	10	−31

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The rate of $[Ca^{2+}]_{ER}$ decay in intact cells can be influenced by factors other than the intrinsic ER Ca^{2+} leak, for example, the rate of Ca^{2+} extrusion across the plasma membrane or the rate of Ca^{2+} sequestration by mitochondria or other organelles. We thus investigated whether the reduction of the steady-state ER Ca^{2+} level, induced by transient expression of mutant PS2, could also be observed in digitonin permeabilized cells with the free $[Ca^{2+}]$ of the bathing solution buffered at a constant level by an EGTA-based buffer (see Materials and Methods). Under these conditions, SH−SY5Y cells cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R showed a reduction of the $[Ca^{2+}]_{ER}$ (compared with controls) similar to that found in intact cells: 53 and 47%, respectively, in intact and permeabilized SH−SY5Y cells (Fig. 2A). Similar results were also obtained in DKO MEFs (Fig. 2B). The capability of PS2-T122R to reduce the $[Ca^{2+}]_{ER}$ level upon cell treatment with digitonin (20−100 μM, 1–2 min.) was confirmed also in an SH-SY5Y clone stably expressing PS2−T122R. The estimated $[Ca^{2+}]_{ER}$ reductions, compared with the void-vector transfect clone, were 25.8 ± 2.9% ($n = 20$, $P < 0.01$) and 28.1 ± 5.4% ($n = 22$, $P < 0.01$), respectively, in intact and permeabilized cells.

PS2-T122R effect on ER Ca^{2+} channels

It has been suggested that, in PC12 cells and in cortical neurons, PS2-N141I (as well as PS1 mutations) alters Ca^{2+} homeostasis

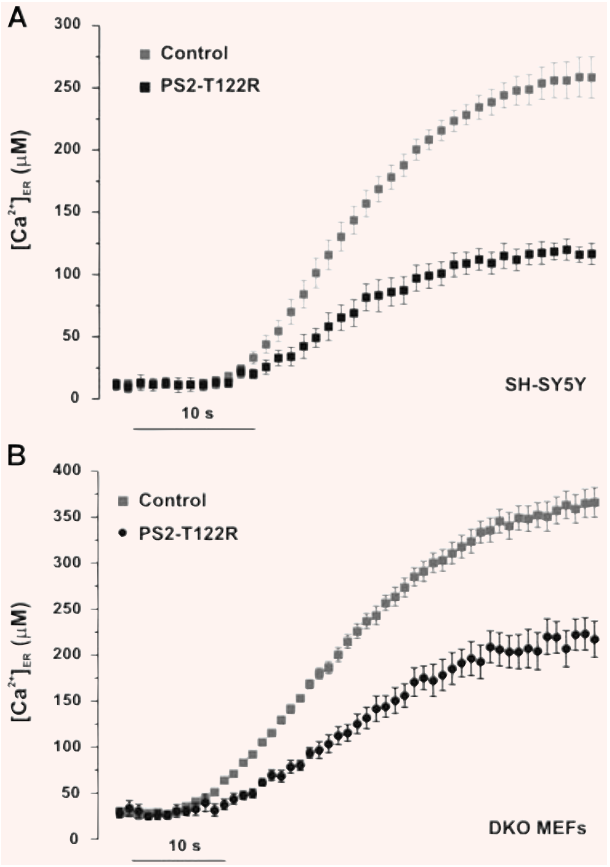

Fig. 2 Effect of PS2-T122R on ER Ca^{2+} uptake. (**A**–**B**) SH-SY5Y cells (**A**) and DKO MEFs (**B**) were transiently cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R (black) or with the void vector as control (grey). Upon Aeq reconstitution (see Materials and Methods), the cells were washed and bathed in a Ca^{2+}-free, EGTA (0.6 mM)-containing medium. The cells were then permeabilized for 2 min. with digitonin (20 μM) in an EGTA-containing intracellular solution. Upon washing, ER Ca^{2+} uptake was followed in the same solution containing an EGTA-based buffer (free $[Ca^{2+}] = 0.3$ μM) and ATP (0.2 mM) (see Materials and Methods). Traces were aligned to Ca^{2+} addition, black and grey symbols for PS2-T122R expressing and control cells, respectively (mean ± S.E.M., $n = 12$ for SH-SY5Y and $n = 16$ for DKO MEFs).

by interfering with ryanodine receptors (RyRs) [2, 9, 10]. Furthermore, it has been shown that, in triple transgenic mice, the knock-in of PS1-M146V up-regulates the expression of RyRs in the brain [5, 13] and in single-channel activity experiments, the cytosolic N-terminus of PS1 or PS2 potentiates mouse RyR open probability [33, 34]. We employed a pharmacological approach to investigate whether RyRs could be possibly involved in the effect of PS2-T122R on ER Ca^{2+} handling. In intact SH-SY5Y cells, an overnight treatment with the classical RyR inhibitor dantrolene (20 μM) caused only a minor reduction of the PS2-T122R effect. In fact, when the Ca^{2+} store content was estimated by cyt-Aeq following the addition of CPA (20 μM) plus bradykinin (Bk, 100 nM) (Fig. 3A), a small but significant recovery of the reduction caused by PS2-T122R was observed (+12%, $P < 0.05$) (Fig. 3B). A similar trend to a recovery, albeit not statistically significant (+5%), was found while monitoring the steady-state ER Ca^{2+} level in dantrolene−treated cells (data not shown). Consistently with the modest effect found upon RyR inhibition, the process of Ca^{2+}-induced Ca^{2+} release seemed not to play a dominant role, since in permeabilized SH-SY5Y cells perfused with an intracellular solution containing BAPTA (a Ca^{2+} chelator acting faster than EGTA) the effect of PS2-T122R on the $[Ca^{2+}]_{ER}$ was similar, being the estimated reductions of ER Ca^{2+} plateaus 39 ± 3% and 34 ± 3% (mean ± S.E.M., $n = 3$), respectively, with BAPTA- and EGTA-based buffers (see Materials and Methods).

IP_3Rs are the other class of Ca^{2+} release channels that have also been involved in the modulation of Ca^{2+} handling by PSs [4, 7, 11, 12, 22]. When the steady-state $[Ca^{2+}]_{ER}$ was monitored in permeabilized SH-SY5Y cells, transfected with ER-Aeq and PS2-T122R, addition of the IP_3R antagonist heparin (100–200 μg/ml) had no effect or caused only a marginal recovery. On average, the reduction in the steady-state was maintained (40 ± 1.4% and 41 ± 6%, mean ± S.E.M., $n = 5$, respectively, with and without heparin). The other IP_3R inhibitor 2−aminoethoxydiphenyl borate (2−APB) could not be used because it interfered with the ER-Aeq–based detection system already at 20 μM, a concentration smaller than that usually employed to block the receptor (unpublished data). In MEFs that, at variance with SH-SY5Y, express high levels of IP_3R-3 in addition to IP_3R-1 [22, 35], knocking down IP_3R-3 by a specific siRNA, partially rescued (+10%, $P < 0.01$) the ER Ca^{2+} defect caused by expression of PS2-T122R in the same interfered cells (Fig. 3C); a siRNA specific for the type 1 IP_3R was ineffective (Fig. 3D). Although siRNAs to both IP_3Rs significantly and potently reduced the specific receptor level, no additional rescue was found (data not shown).

We also investigated another possible ER exit pathway for Ca^{2+} ions. It has recently been suggested that the protein import machinery across the ER membrane could be a relevant Ca^{2+} leak pathway. In particular, it has been shown that the protein import machinery, the so-called ribosomal–translocon complex (RTC), once freed of the newly synthesized protein, can allow the flux of Ca^{2+} ions and small sugars [36–38].

When SH-SY5Y cells were maintained in the presence of puromycin (200 μM), following a 10-min. pre-incubation at 37°C, a protocol sufficient to release nascent proteins and leave the RTC in an open configuration [36], the steady-state $[Ca^{2+}]_{ER}$ was unaffected both in control and PS2-T122R–expressing cells (Fig. 4A). When a similar treatment was also employed in control SH-SY5Y cells expressing cyt-Aeq, the rate of store Ca^{2+} efflux ($d[Ca^{2+}]_{cyt}/dt$) induced by CPA addition was of the same magnitude with or without puromycin treatment (9.6 ± 0.5 nM/sec. and 8.9 ± 1.0 nM/sec., mean ± S.E.M., $n = 3$). The involvement of the RTC as a possible target of PS action was also investigated by employing anisomycin. This peptidyl-transferase inhibitor locks nascent chains in the ribosome, leaving the RTC in a closed

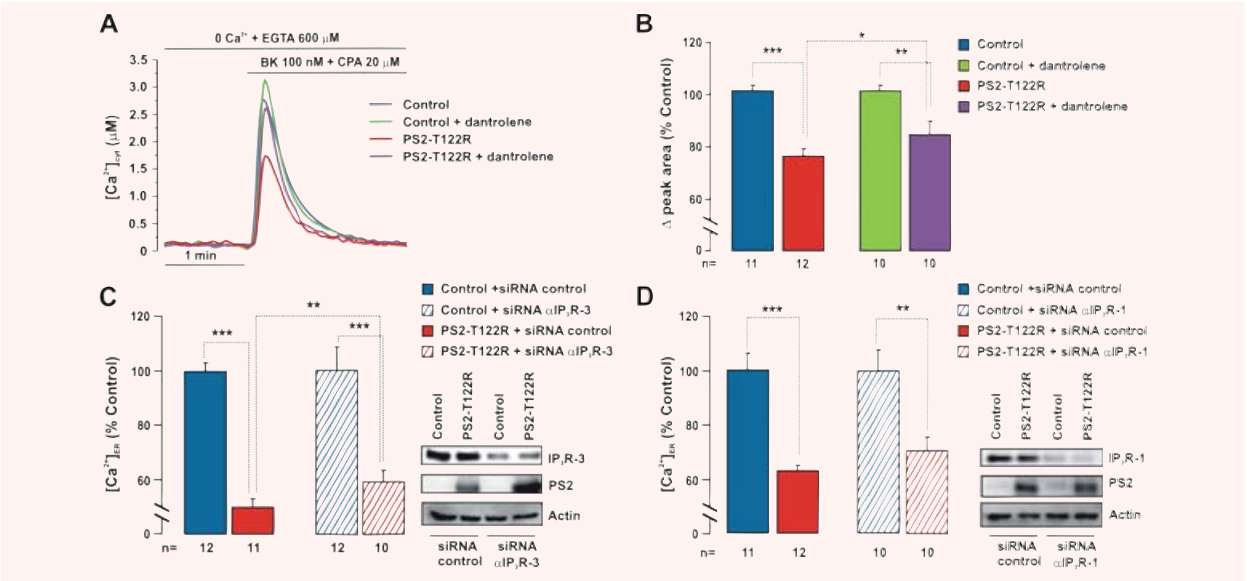

Fig. 3 Role of RyRs and IP_3Rs. (**A**) SH-SY5Y cells, transiently cotransfected with the cDNAs coding for cyt-Aeq and PS2-T122R (red trace), or with the void vector, control (blue trace), were overnight treated with dantrolene (20 μM) and continuously maintained in the presence of the drug (purple and green traces, respectively). Upon Aeq reconstitution (see Materials and Methods), the cells were washed in mKRB and then exposed to CPA (20 μM) plus bradykinin (Bk, 100 nM) in a Ca^{2+}-free, EGTA (0.6 mM)-containing medium to fully discharge the intracellular Ca^{2+} stores. (**B**) Bars represent the average peak area measured above the baseline and expressed as percentage of control, void-vector transfected cells (mean ± S.E.M.). (**C**, **D**) wt MEFs were cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R (or with the void vector as control) and siRNA specific for mouse IP_3R-3 (40 nM) (**C**), IP_3R-1 (20 nM) (**D**) or equivalent amounts of control siRNA (**C**, **D**). After 48 hrs, cells were harvested and probed for expression levels of PS2 and IP_3Rs by Western blotting (right) or were employed to measure steady-state ER Ca^{2+} levels (left), according to the protocol described in Fig. 1A. Bars represent the average $[Ca^{2+}]_{ER}$ expressed as percentage of control cells transfected with the void-vector and control siRNA (mean ± S.E.M.). Note interruption in the *Y* axes in (**B**)−(**D**).

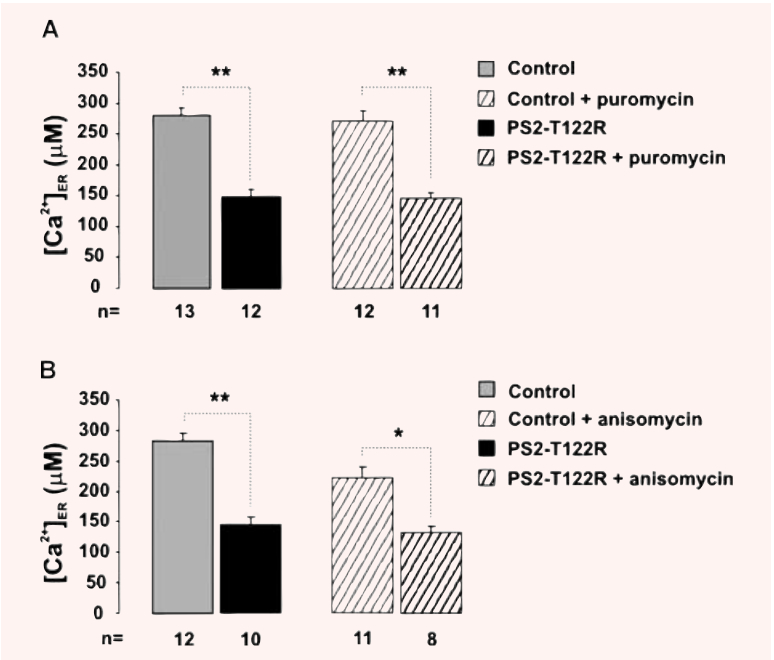

Fig. 4 Role of the ribosomal–translocon complex. (**A**) SH-SY5Y cells were transiently cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R (or with the void vector as control). Upon ER-Aeq reconstitution, cells were treated for 10 min. with puromycin (0.2 mM) at 37°C and continuously maintained in the presence of the drug. ER Ca^{2+} levels were measured as described in Fig. 1A. (**B**) SH-SY5Y cells were transfected as described in (**A**). Anisomycin (0.2 mM) was present during ER-Aeq reconstitution and throughout the experiments. Bars represent the average $[Ca^{2+}]_{ER}$ (μM) (mean ± S.E.M.).

configuration. Anisomycin was reported to prevent opening of the RTC if added simultaneously or before puromycin [36]. As shown in Fig. 4B, incubation for 1 hr at 4°C with anisomycin (200 μM) during the ER-Aeq reconstitution protocol, and continuous exposure to the drug during the experiment, did not prevent the reduction of the ER Ca^{2+} level, induced by PS2-T122R expression in SH-SY5Y cells. Interestingly, treatment with this drug, that should close a passive ER Ca^{2+} leak pathway, decreased the steady-state ER Ca^{2+} level in the control cells (–22 ± 6.8%, $n = 6$, $P < 0.05$).

Based on indirect evidence, that is, cytosolic Ca^{2+} sequestration, LaFerla and colleagues recently suggested that wt PS1 and PS2, as well as an FAD mutant PS1 (M146V), increase SERCA activity [25]. To directly address whether and how PS2 affects the activity of SERCA pumps, we carried out a detailed analysis in permeabilized SH−SY5Y cells by means of ER-Aeq. Ca^{2+} uptake rates were measured in different ranges of free [Ca^{2+}], set by distinct Ca^{2+}-EGTA–based buffers (see Materials and Methods). The maximal values of the first derivative of the instantaneous $[Ca^{2+}]_{ER}$ ($d[Ca^{2+}]_{ER}/dt$) were plotted as a function of the imposed free $[Ca^{2+}]_0$ (Fig. 5A). Expression of PS2-T122R significantly and consistently reduced the maximal rate of Ca^{2+} uptake, at all the [Ca^{2+}] tested. Similar reductions in ER Ca^{2+} uptake were observed in DKO MEFs (Fig. 5B) and wt MEFs (data not shown) expressing PS2-T122R, upon cell permeabilization with digitonin (20 μM). Table 2 reports the estimated K_m and V_{max} values of Ca^{2+} uptake obtained by double-reciprocal plots; in all cell types, the trend was similar: expression of PS2-T122R strongly reduced the V_{max} (22–35%) with modest increases in the K_m (7–16%). Noteworthy, in DKO MEFs, the ER Ca^{2+} uptake rates were also similarly reduced upon expression of wt PS2 (12.2 ± 0.9 μM/sec. and 9.4 ± 0.9 μM/sec., for control and wt PS2–expressing cells with the free [Ca^{2+}] buffered at 300 nM; mean ± S.E.M., $n = 7$, $P < 0.05$; Fig. 5B, triangles). It should be stressed that these values represent the initial rates of the Ca^{2+} refilling process, that is, when the PS2 effect on leak rates should be negligible, being the ER lumen practically empty of Ca^{2+}. Differences in maximal uptake rates were maintained even in the presence of heparin (200 μg/ml; data not shown).

Because expression of PS2-T122R reduces the ER Ca^{2+} uptake without reducing the SERCA-2 protein level (Fig. 5C), the simplest interpretation of these results is that PS2 variants affect directly or indirectly the activity of the pump. Experiments were carried out to determine whether the ER Ca^{2+} depletion induced by PS2-T122R could be compensated by increasing the number of SERCA-2B. Figure 5D and E shows the results obtained by monitoring ER Ca^{2+} levels in digitonin permeabilized SH-SY5Y cells: over-expression of SERCA-2B together with PS2-T122R allowed to fully recover both the maximal uptake rate and the ER Ca^{2+} plateau found in control cells.

Conformation of PS2 and store Ca^{2+} handling

It has been reported that only the full-length (FL), immature forms of wt PSs (but not FAD-linked mutants) can form ER Ca^{2+} leak channels [23, 26] (but see also [11]). We asked which form of the protein, the FL or the dimeric complex formed by the N- and C-terminal fragments (NTF, CTF), is responsible for the reduced ER Ca^{2+} level here reported. In DKO MEFs, transient expression of the loss-of-function mutant PS2-D366A, which is devoid of γ-secretase as well as presenilinase activity [39] (Fig. 6A), reduced the ER Ca^{2+} level by an amount similar to that observed upon expression of wt or mutant PS2 (Fig. 6B).

Lendahl and colleagues [40] have previously demonstrated that, in DB8 cells KO for both PSs, co-expression of the NTF and CTF of wt PS2, by means of a bicistronic vector, allows the recovery of γ-secretase activity. We confirmed this result in DKO MEFs by employing a recently developed γ-secretase cell assay [30] (Fig. 6C); NTF and CTF co-expression, however, failed to mimic the effect of wt or mutant PS2 on Ca^{2+} handling (Fig. 6B).

Role of endogenous PS2 on ER Ca^{2+} uptake

All the above results were obtained in cells over-expressing PS2. To test whether physiological PS2 levels play a role in ER Ca^{2+} handling, wt MEFs were cotransfected with siRNA specific for mouse PS2 and the cDNA coding for ER-Aeq. As shown in Fig. 7, the protein level was reduced by 50–80%, 24–48 hrs after transfection (see Materials and Methods). When ER Ca^{2+} plateaus and uptake rates were evaluated at this time-point, upon digitonin permeabilization, a small but significant increase in both parameters was detected in cells knocked down for PS2, compared with cells treated with control siRNA (Fig. 7A and B). On the other hand, when cells were transfected with the cDNA coding for ubiquilin1 [41] (Fig. 7C) or were treated overnight with the presenilinase inhibitor MW167 (15 μM) [42] (Fig. 7D), both treatments known to increase FL PS levels by interfering with PS processing, significant decreases of ER Ca^{2+} plateaus were observed in wt but not in DKO MEFs.

We had previously shown that, in HeLa and SH-SY5Y cells as well as in human FAD fibroblasts, PS1 mutants partially mimicked the ER Ca^{2+}–depleting effect of PS2 mutants [18]. We here evaluated the hypothesis that the former mutants can exert an ER-depleting effect only in the presence of PS, that is, in an endogenous PS background. In fact, the transient expression of PS1-A246E significantly decreased the ER Ca^{2+} plateau by 25% in wt MEFs (243 ± 34 μM and 325 ± 21 μM, mean ± S.E.M., $P < 0.05$, $n = 9$) but not in DKO MEFs (270 ± 28 μM and 314 ± 20 μM, mean ± S.E.M., $n = 9$; Fig. 7E).

Discussion

We have previously reported that different FAD-linked PS1 and PS2 mutants instead of causing a Ca^{2+} overload, *reduce* the ER Ca^{2+} content in different model cells, including fibroblasts from FAD patients and rat primary neurons. The effect is consistent and more dramatic with PS2 variants, and it is also mimicked by

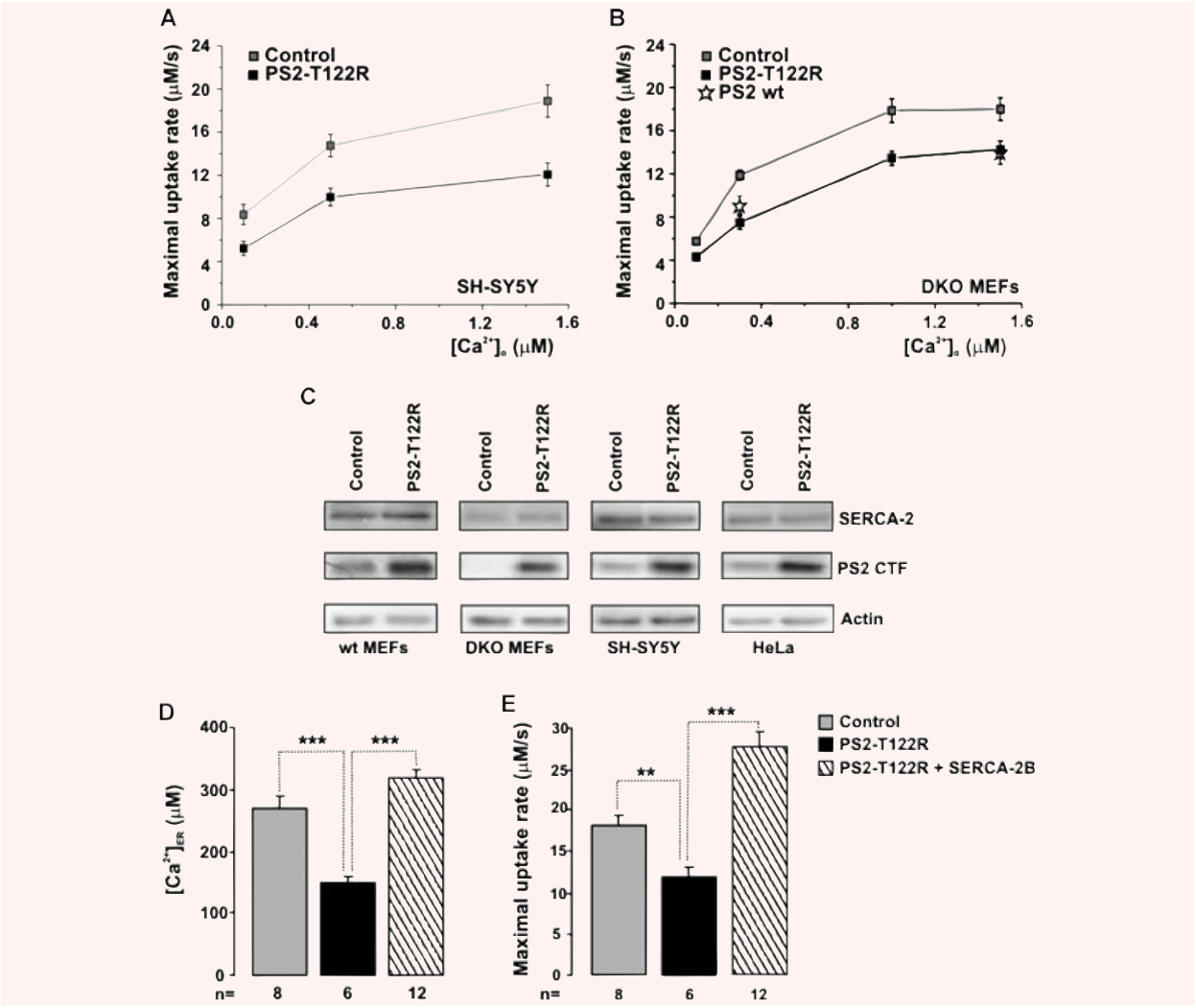

Fig. 5 Effect of PS2-T122R on ER Ca^{2+} uptake. (**A**, **B**) ER Ca^{2+} uptake was followed in permeabilized SH-SY5Y cells and DKO MEFs at different free [Ca^{2+}] as described in Fig. 2. The maximal values of the first derivative of the instantaneous $[Ca^{2+}]_{ER}$ ($d[Ca^{2+}]_{ER}/dt$) were plotted as a function of the free external $[Ca^{2+}]_o$ (mean ± S.E.M., *n* ranging from 6 to 20). (**B**) also shows the values obtained with PS2 wt (empty stars): 9.4 ± 0.9 μM/sec. ($n = 7$) and 14.4 ± 0.8 μM/sec. ($n = 8$), respectively, at 0.3 and 1.5 μM $[Ca^{2+}]_o$. (**C**) Western blots showing SERCA-2 and PS2 levels in control and PS2-T122R–expressing cell lines. (**D**, **E**) SH-SY5Y cells were transiently cotransfected with the cDNAs coding for ER-Aeq and PS2-T122R in the absence (black bars) or presence of SERCA-2B cDNA (hatched bars); control cells were cotransfected with ER-Aeq cDNA and with the void vector (grey). Bars represent the average $[Ca^{2+}]_{ER}$ (μM) (**D**) and maximal uptake rates (μM/sec.) (**E**) (mean ± S.E.M.).

Table 2 Kinetic parameters of ER Ca^{2+} uptake

	K_m (nM)		% change	V_{max} (μM/sec.)		% change
Cell type	**Control**	**PS2-T122R**		**Control**	**PS2-T122R**	
SH-SY5Y	150	167	11	19.3	12.8	−34
wt MEFs	164	175	7	14.5	10.1	−30
DKO MEFs	277	321	16	21	16.4	−22

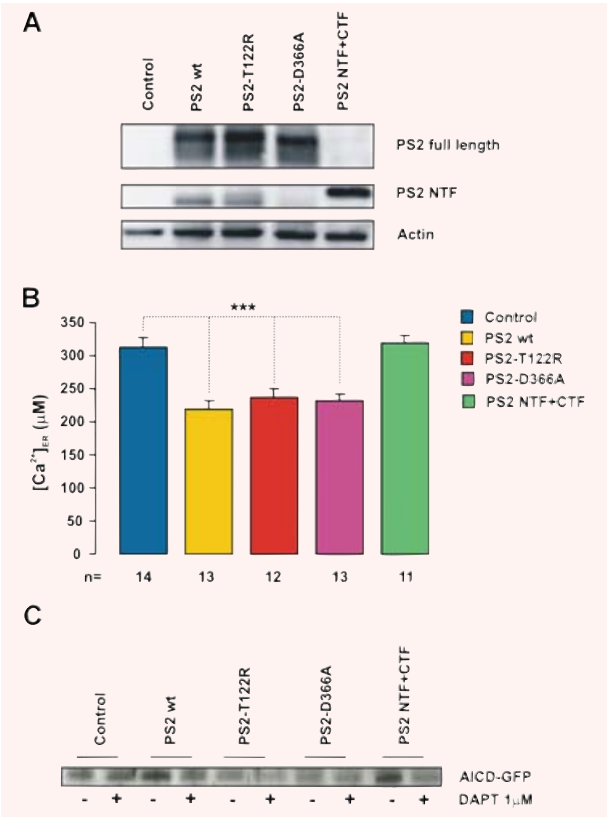

Fig. 6 Effect of PS2 conformation on ER Ca^{2+} levels. (**A**) DKO MEFs were cotransfected as described in Fig. 1A with the cDNA coding for ER-Aeq and a PS2 variant (wt, T122R or D366A) or with a bicistronic vector coding for the NTF and CTF of wt PS2. The Western blot shows the expression level of PS2 (FL and NTF). The PS2 NTF of cells transfected with the bicistronic vector migrates at higher MW because of a myc-tag. (**B**) Bars represent the average $[Ca^{2+}]_{ER}$ (μM) for DKO MEFs described in (**A**) (mean ± S.E.M.). (**C**) The γ-secretase activity was checked in DKO-MEFs transfected with the cDNA coding for C99-GFP as suitable substrate. Co-expression of wt PS2, PS2-T122R or PS2 NTF+CTF, but not PS2-D366A, led to AICD-GFP generation, as detected by Western blot with an anti-GFP antibody. Control cells, transfected as earlier, were overnight treated with DAPT (1 μM).

over-expression of wt PS2 [18, 20, 21]. Similar conclusions have also been reached in neuroblastoma cells by over-expression of the wt forms of PS2 and PS1 [19]. Mild reductions of store Ca^{2+} levels rather than Ca^{2+} overloads were also recently reported in DT40 cells expressing PS1−M146L [11]. Taken together, these data, while questioning the 'Ca^{2+} overload' hypothesis [15, 43], strongly suggest that PSs might be key determinants in setting the ER Ca^{2+} level. We here investigated the molecular mechanism by which wt and mutant PS2 reduce the ER Ca^{2+} content of intracellular stores. To this aim, in addition to HeLa cells, we also used neuroblastoma SH-SY5Y cells and two MEFs clones, with and without endogenous PSs (wt and DKO MEFs, respectively).

When the effect of the transient expression of PS2-T122R was taken as a reference to compare the different cell types, we invariably observed a reduction in the steady-state $[Ca^{2+}]_{ER}$, ranging from about 50% to 20%, with the following efficacy order: SH-SY5Y > wt MEFs ≥ DKO MEFs > HeLa cells. All these models were thus employed to untangle the likely common mechanisms that underlie the PS2 effect.

By means of ER-Aeq, we initially verified whether the amount of passive ER Ca^{2+} leak was increased by expression of wt and mutant PS2. In the absence of Ca^{2+} and in the presence of a SERCA inhibitor, the decay rate of the $[Ca^{2+}]_{ER}$ was modestly but significantly accelerated by expression of PS2-T122R. The effect was more pronounced in SH-SY5Y (+17%) than HeLa cells (+9%). Of note, an increased leakage was also found upon expression of PS2-M239I or wt PS2.

Passive ER leak may be accounted for by classical Ca^{2+} release channels. Indeed, PS2 has been suggested to increase number and/or sensitivity of both RyRs [9, 10, 34, 44] and IP_3Rs [7, 11, 12, 22]. In SH-SY5Y and wt MEF cells, where the PS2 effect was more pronounced, pharmacological (dantrolene and heparin) and genetic (siRNA) approaches, allowed us to estimate an increase in leak, due to both RyRs and IP_3Rs (type 3), that corresponds to about 15%, a value not far from that found in HeLa and SH-SY5Y cells by measuring passive ER Ca^{2+} leakage in the presence of CPA (ranging from 9% to 17%).

We also verified that PS2 did not exert its effect by acting on the protein import machinery, the so-called RTC, which was recently suggested to represent a relevant ER Ca^{2+} efflux pathway [36–38]. Puromycin, a known RTC opener [36], was not able to mimic the PS2 effect on both the cytosolic and ER Ca^{2+} levels of SH-SY5Y cells. Notably, puromycin by itself did not increase the rate of Ca^{2+} exit from the stores. In contrast, anisomycin, a drug that should keep the RTC closed, did not rescue the ER Ca^{2+} loss caused by mutant PS2. Taken together, these data indicate that, at least in SH-SY5Y cells, the RTC does not significantly contribute to the resting ER Ca^{2+} leakage or to that induced by expressed PS2.

We also verified whether there was an effect of PS on ER Ca^{2+} uptake. In intact cells, uptake rates do not simply reflect pump activity, as they are also affected by Ca^{2+} influx/extrusion processes across the plasma membrane and mitochondria Ca^{2+} buffering. To overcome these uncertainties, studies were thus carried out in digitonin permeabilized cells with the free $[Ca^{2+}]$ of the bathing medium buffered at different values (0.1–2 μM). At these Ca^{2+} concentrations, the contribution of mitochondria to Ca^{2+} uptake is negligible. Under these conditions, in cells expressing PS2-T122R, reductions of Ca^{2+} uptake rates were found at each Ca^{2+} concentration tested. Similar results were also obtained in DKO MEFs expressing wt PS2. In SH-SY5Y, over-expression of SERCA-2B together with PS2-T122R rescued both ER Ca^{2+} uptake rates and steady-state levels at, or above, the values observed in control, void-vector transfected cells. Recently, Green *et al.* [25] have shown that wt PS1 and PS2 as well as an FAD-linked mutant (PS1-M146V) increase SERCA-2 activity. Those data, however, have been obtained by monitoring cytosolic Ca^{2+} clearance in intact *Xenopus* oocytes, that is, under conditions

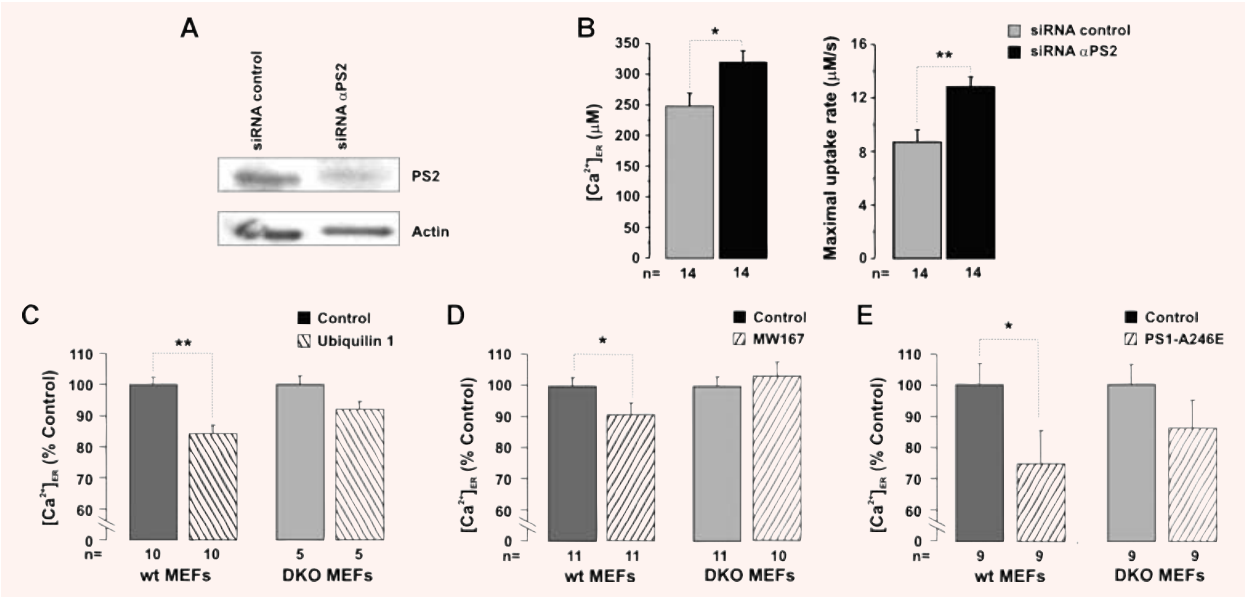

Fig. 7 Effect of endogenous PS2 on ER Ca^{2+} uptake. (**A**) wt MEFs were transfected with the cDNA coding for ER-Aeq and siRNA specific for mouse PS2 or control siRNA (20 nM). After 48 hrs, part of the cells was harvested to check the expression level of PS2 by Western blotting. (**B**) The same cells were used to estimate ER Ca^{2+} uptake upon cell permeabilization with the protocol described in Fig. 2. Bars represent the average $[Ca^{2+}]_{ER}$ (μM) (left) and maximal uptake rates (μM/sec.) (right) (mean ± S.E.M.). (**C**) wt and DKO MEFs were transfected with the cDNA coding for ER-Aeq and ubiquilin1. (**D**) wt and DKO MEFs were transfected with the cDNA coding for ER-Aeq and overnight treated with MW167 (15 μM) or vehicle (DMSO). (**E**) wt and DKO MEFs were transfected with the cDNA coding for ER-Aeq and PS1-A246E or its void vector (control). Bars represent the average $[Ca^{2+}]_{ER}$ (mean ± S.E.M.) expressed as percentage of control cells transfected with the void vector (**C**, **E**) or treated with vehicle (**D**); note interruption in the *Y* axes. (**C**, **E**) Upon Aeq reconstitution, steady-state ER levels were measured as described in Fig. 1A.

where other factors such as the amplitude of capacitative Ca^{2+} influx, the activity of the plasma membrane Ca^{2+} ATPase and the contribution of mitochondrial Ca^{2+} uptake may complicate data interpretation. From our results obtained in permeabilized cells, we could conclude that PS2-T122R affects the maximal capacity of the pump rather than its K_m, which appears to be set around 0.15–0.3 μM, a value not far from that reported for purified SERCA-2B (0.17 μM) [45].

With these results in mind, one would expect to find ER Ca^{2+} overloads in DKO MEFs, if compared with wt MEFs. On the contrary, we found similar ER Ca^{2+} steady-state levels and about a 20% increase in ER Ca^{2+} uptake rates in permeabilized DKO MEFs. It should be noted that previous data using these model cells found both ER Ca^{2+} overload [23] and ER Ca^{2+} reduction [22, 25], despite the fact that these clones all derive from the same laboratory (B. De Strooper's lab; [31]). The different results do not depend on different approaches to test Ca^{2+} handling, since both the fura-2 [23, 25] and recombinant aequorin [22] were used, as we did. Moreover, we could not confirm in the DKO MEFs differences in the expression levels of IP_3Rs [22] or SERCAs [25]. These discrepancies indicate that, despite originating from the same transgenic mice, cell clones can substantially differ one from the other (at least in terms of Ca^{2+} handling) and, more important, that a cause–effect relationship between alterations in Ca^{2+} homeostatic machinery and lack of PS expression cannot be unambiguously established using these cells. On the contrary, such a cause–effect relationship appears more easily and consistently found upon transient expression of wt or mutant PS1/2 in each clone. We followed this approach to test the hypothesis that endogenous PS2 works as a brake on ER Ca^{2+} uptake. Along the same line, in wt MEFs, knocking down the endogenous level of PS2 by siRNAs increased both ER Ca^{2+} pumping and steady-state levels, thus unmasking the inhibitory role played by PS2.

In DKO MEFs, the effect of mutant PS2 on ER Ca^{2+} handling does not qualitatively differ from that exerted by either wt PS2 or PS2-D366A, a loss-of-function, non-pathogenic mutant also devoid of presenilinase activity (see Fig. 6A and [39]). These findings suggest that PS2 and possibly SERCA-2 interact independently of the γ-secretase activity and the intra-molecular cut of PS2. To further address this issue, we have co-expressed the NTF and CTF of wt PS2 in DKO MEFs using a bicistronic vector [40]. Expression of the two separate fragments, while being able to rescue the γ-secretase activity, was unable to mimic the effect of PS2 on ER Ca^{2+} handling. Furthermore, the Ca^{2+}-depleting effect of PS2 over-expression could be partially mimicked by ubiquilin1 over-expression [41] or inhibition of presenilinase activity by

MW167 [42], two treatments that have been previously suggested to stabilize the FL form of endogenous PS2.

We previously found that transient or stable expression of some FAD-linked PS1 mutants also causes ER Ca^{2+} depletion. This effect, however, is quantitatively much smaller than that caused by PS2 expression and is dependent on the type of mutation (A246E, M146L, P117L but not L286V; [18]) and the cell system employed. For example, PS1-A246E was effective in HeLa cells and wt MEFs but not in SH-SY5Y cells and primary rat neurons (this work and [18]). These results might be explained taking into account recent findings that suggest the capability of PS1 mutants to shift the balance from PS1 to PS2 containing γ-secretase complexes [46]. Thus, given the inhibitory role played by endogenous PS2 on ER Ca^{2+} uptake, the store-depleting effect of some PS1 mutants could be indirect and reside in their capability to potentiate the effect of endogenous PS2, either as single molecule or as part of a complex. This is, however, only a working hypothesis, and additional experiments are required to address this specific point.

In conclusion, we here provide evidence showing that PS2 has a dual role on ER Ca^{2+} homeostasis: (*i*) it inhibits SERCAs and (*ii*) increases Ca^{2+} leak through ER Ca^{2+} channels (RyRs and IP_3R-3). The effect on Ca^{2+} uptake was dominant in SH-SY5Y with respect to HeLa cells. This finding is consistent with the fact that cells of the former type, like neurons, have very low resting ER Ca^{2+} levels. Altogether, these results suggest that PS2 plays a direct, primary role on Ca^{2+} handling by intracellular stores while strengthening the idea of a complex interplay between PS1 and PS2.

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Current Trends in Neurology

Review

Ca^{2+} dysregulation mediated by presenilins in Familial Alzheimer's Disease: Causing or modulating factor?

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ABSTRACT

Ca^{2+}, one of the major intracellular messengers, plays essential roles in neuronal development, synaptic transmission and plasticity, as well as in the regulation of metabolic pathways. A perturbed Ca^{2+} homeostasis has been demonstrated in Alzheimer's Disease (AD), one of the most devastating neurological disorder of the elderly. Although the majority of AD cases are sporadic, a small fraction is inherited in a dominant pattern (Familial AD, FAD). Of the three genes involved in the pathogenesis of FAD, two code for the ubiquitously expressed proteins presenilin (PS) 1 and 2. Mutations in PSs have variably been correlated to alterations of Ca^{2+} signalling and different molecular targets have been identified, suggesting a physiological role for these proteins in multiple intracellular Ca^{2+} pathways. According to the popular "Ca^{2+} overload" hypothesis for FAD pathogenesis, PS mutations increase the Ca^{2+} content of the endoplasmic reticulum (ER), thus sensitizing neurons to excitotoxicity and progressive degeneration. The latter process is closely linked to exaggerated ER Ca^{2+} release that, in turn, causes abnormal mitochondrial Ca^{2+} uptake and cell death. New evidence from different groups has however shown exceptions to this scenario: in fact, in addition to an increased, also a reduced, or even an unchanged, ER Ca^{2+} content has been described in cells over-expressing wild type or FAD mutant PSs. Altogether, these findings suggest that Ca^{2+} dysregulation in FAD is variable in nature, depending on the type of mutation and the cell model under investigation, thus appearing as a modulator rather than a causing event in AD pathogenesis.

KEYWORDS: presenilin, calcium, Alzheimer's Disease

INTRODUCTION

The control of Ca^{2+} homeostasis is central to many physiological processes such as muscle contraction, active secretion, cell differentiation and death [1, 2]. The role of Ca^{2+} ions as key mediators in cell activation is particularly important in the central nervous system (CNS), and it is undisputed that processes such as neurotransmitter release from synaptic terminals, long term modification of synaptic strength or excitotoxicity, just to mention a few, are strictly dependent on changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In both excitable and non-excitable cells, cytosolic Ca^{2+} rises depend on Ca^{2+} release from intracellular stores and/or Ca^{2+} influx across the plasma membrane, though the importance of these processes can vary depending on the cell type and the stimulus. As to the latter mechanism, tens of different forms of Ca^{2+} permeable channels are expressed on the plasma membrane of all cells. The specific repertoire of Ca^{2+} channels depends on the cell type, the developmental level and the subcellular domain of the plasma membrane [3]. Ca^{2+} release from intracellular organelles is most often triggered by

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the activation of phospholipase C (different isoforms), that produces inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol. IP_3 interacts with its receptors (IP_3Rs), Ca^{2+} channels localised in the endoplasmic reticulum (ER) and Golgi apparatus, causing their opening and the release of Ca^{2+} into the cytosol. Another intracellular Ca^{2+} release pathway relays on ryanodine receptors (RyRs), particularly expressed in muscle cells. RyRs are large tetrameric channels that span the ER or the sarcoplasmic reticulum. The primary natural agonist of RyRs is Ca^{2+}; a modest increase in the $[Ca^{2+}]_i$ (μM) opens the channel and allows Ca^{2+} to flow out of the ER/sarcoplasmic reticulum, while a higher rise near the mouth of the channel inhibits gating and prevents Ca^{2+} overload [4].

Ca^{2+} release from stores is most often accompanied by Ca^{2+} influx through Capacitative Ca^{2+} Entry, (CCE), whose nature and mechanism of opening have recently been defined (see [5] for a recent review). The Ca^{2+} signal is terminated by the combined activities of the Ca^{2+} extrusion mechanisms: the plasma membrane Ca^{2+} ATPase and the Na^+/Ca^{2+} exchanger. The sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) and the secretory pathway Ca^{2+} ATPase1 (SPCA1) of the Golgi apparatus cooperate by accumulating the cation inside the organelle lumen [2]. The intracellular Ca^{2+} signalling is extremely versatile in terms of speed, amplitude and spatio-temporal patterns. At this level, the specific ability of different intracellular organelles (endosomes, lysosomes, secretory granules and mitochondria) in accumulating and buffering Ca^{2+} should also be considered [6].

Recent evidence indicates that Ca^{2+} handling mechanisms, particularly of mitochondria and ER which play a key role in the process of cell death, are impaired in many degenerative processes of the CNS, such as Huntington, Parkinson, Amiotrophic Lateral Sclerosis and AD [7]. A general consensus has been reached supporting the idea that extensive Ca^{2+} accumulation by mitochondria, particularly, but not only, in response to Ca^{2+} release from the ER, possibly concomitant with other toxic insults, initiates the release from mitochondria of proapoptotic proteins that eventually lead to the activation of executor caspases [8-10].

As far as AD is concerned, the idea that a Ca^{2+} dysregulation is involved in the etiopathology is a long standing hypothesis based on the observations that peripheral cells from AD patients show increased [11-13] as well as decreased [14-20] Ca^{2+} responses, as compared to control cells, upon different stimulation protocols. Moreover, a disturbed Ca^{2+} homeostasis has been suggested to be an early event in AD since evidence from human subjects and from experimental models shows that alterations in Ca^{2+} signalling occur during the initial phases of the disease, even before the development of overt symptoms [21, 22] or the presence of extracellular Aβ pathology [23, 24].

The most powerful model of AD pathogenesis is based on the “amyloid hypothesis” which states that synaptic loss, cognitive deficit and neuronal death are all caused by increased ratios of Aβ42 to Aβ40 peptides [25]. Aβ peptides (especially Aβ40 and Aβ42, the most abundant forms) are the principal components of the amyloid (senile) plaques that massively accumulate in the brain of AD patients. Aβ peptides derive from the processing of the transmembrane protein Amyloid Precursor Protein (APP) by three different enzymes, named α, β, and γ secretases. The combined action of β and γ secretases leads to the formation of a soluble fragment (sAPPβ) and of Aβ, together with its cytosolic counterpart, the APP Intra-Cellular Domain (AICD) [26].

Although the large majority of AD cases are sporadic, a small but significant fraction of them is inherited in a dominant pattern. Mutations in the genes encoding for APP and PS1 (or PS2), a protein belonging to the γ-secretase complex, have been linked to FAD [27]. Since the majority of FAD mutations have been found to increase the Aβ42/Aβ40 ratio, the suggested hypothesis was that the disease is dependent on the enhanced oligomerization of the more amyloidogenic Aβ42 peptide. It was starting from the observation that many FAD mutations in PSs result in abnormal Ca^{2+} signalling (see below) that an additional hypothesis for AD pathogenesis was brought about: the so called “Ca^{2+} hypothesis”. From a mechanistic point of view, its most accepted scheme relies on the following: in FAD, neuronal degeneration depends on exaggerated or

supranormal ER Ca^{2+} release that in turn leads to abnormal mitochondrial Ca^{2+} uptake and apoptosis [28, 29]. Elevated cytosolic Ca^{2+} concentrations also lead to activation of the Ca^{2+}-dependent phosphatase calcineurin and hence to neurite atrophy, synaptic loss and cognitive deficit [30].

In this review we simply focus on the mechanisms through which FAD-linked PS mutants alter Ca^{2+} homeostasis and how this might be involved in the pathogenesis and progression of the disease.

FAD-presenilins and Ca^{2+}: The overload hypothesis

PS, present as two homologous proteins PS1 and PS2, is an essential component of the APP cleaving enzyme γ-secretase [31]. As an integral membrane protein, it is abundantly expressed with markers of ER, Golgi apparatus and endo-exocytic vesicles, however, only a minor fraction is present at the plasma membrane level [32, 33]. In the mature complex of the γ-secretase, it is found as two fragments (the C- and N-terminal) formed by an endoproteolytic cleavage. Both PS1 and PS2 are now regarded as multi-faceted proteins whose roles can be functionally distinguished and independent of their enzymatic activity [34].

Several lines of evidence suggest that PS is also involved in the regulation of cellular Ca^{2+} homeostasis, either as a component of the enzymatic complex or as an independent protein. Since their discovery, different FAD-linked PS mutants were reported to induce alterations of cellular Ca^{2+} handling in several cell models (see Table 1). In particular, it was observed that the FAD-mutant PS1-L286V, when expressed in PC12 cells, causes an increased Ca^{2+} release upon different stimulations [35-38]. Then, other two FAD mutations in PS1 (A246E and M146V) were described to cause larger Ca^{2+} release from intracellular stores and increase excitotoxicity in neurons from transgenic (Tg) mice expressing the mutant protein [23, 39-43]. The idea that FAD-linked PS mutations are somehow correlated to altered Ca^{2+} signalling was further supported by the fact that these mutations could modify the sensitivity (or expression) of ER Ca^{2+} release channels (RyR and IP_3R) in different cell models [35, 39, 44-50], in neurons from Tg AD mice [42, 43, 51, 52] as well as in isolated brain microsomes [53, 54]. Interestingly, similar observations were done also in non neuronal cells, such as fibroblasts, lymphocytes and *Xenopus* oocytes, indicating that PS plays a central role in Ca^{2+} homeostasis [24, 50, 55]. Moreover, it has been suggested that PS mutants affect also one of the main Ca^{2+} entry pathway, CCE [24, 56-60], although the mechanism, as well as the correlation with the increased Ca^{2+} release is not clear (but see also [61]).

Effects on Ca^{2+} homeostasis were found, albeit of opposite sign, also in PS knock-out (KO) models, suggesting that the endogenous wild type (wt) PS might play a direct role in Ca^{2+} signalling. In particular, it has been reported that left ventricular papillary muscles of PS2 KO mice show enhanced Ca^{2+} transients and contractility [62], whereas primary cortical neurons and hippocampal slices from PS1 KO mice present impaired glutamate-evoked Ca^{2+} responses, altered activity of voltage-gated Ca^{2+} channels and increased LTP due to CCE activation [59, 63, 64]. In B lymphocytes, lacking PS1 and PS2, decreased Ca^{2+} responses to IgM were observed [65], instead mouse embryonic fibroblasts (MEFs) double knocked out (DKO) for both PS1 and PS2 [66] gave contradictory results: in fact, either similar [67, 68], overloaded [69] or even reduced ER Ca^{2+} content [49, 70] was reported, indicating that, although originating from the same mice [66], cell clones can substantially differ one from the other (at least in terms of Ca^{2+} handling) and, more importantly, that a cause-effect relationship between alterations in the Ca^{2+} homeostatic machinery and lack of PS expression cannot be unambiguously established by simply comparing these cell clones. Nonetheless, they represent invaluable tools for expression of different PS forms in a null background [71, 72].

Despite these incongruities and, more importantly, in contrast to recent data (see below), the "Ca^{2+} overload" hypothesis is actually the only "Ca^{2+} hypothesis" for AD pathogenesis, stating that FAD-linked PS mutations, by increasing the store Ca^{2+} content, cause excessive Ca^{2+} release from the ER, increase sensitization to Aβ and exo-citotoxic stimuli and eventually lead to cell death via Ca^{2+}-dependent mechanisms [28, 29, 73].

Table 1. Effects of presenilins on Ca^{2+} homeostasis (I).

Presenilin mutations	**Cell models**	**Methods**	**Effects**	**Ref.**
Sporadic AD/ageing	Fibroblasts	Quin-2, Fura-2	< cytosolic Ca^{2+}	18
Sporadic AD	Fibroblasts	Fura-2	< Ca^{2+} release	19
FAD/sporadic AD	Fibroblast	Aequorin	< Ca^{2+} release	15
Sporadic AD	Fibroblasts	Fura-2	> Ca^{2+} release; = ER Ca^{2+};	12
Sporadic AD, Familial AD (APP)	Fibroblasts	Indo-1	< Ca^{2+} release	17
PS1-L286V	PC12 cells	Fura-2	> Ca^{2+} release	35
Sporadic AD	Fibroblasts	Fura-2	> Ca^{2+} release	11
PS1-L286V	PC12 cells	Fura-2	> Ca^{2+} release	36
APP670/671	Fibroblasts	Fura-2	< Ca^{2+} release	20
PS1-L286V, PS1-M146V	PC12 cells	Fura-2	> Ca^{2+} release	82
PS1-L286V	PC12 cells	Fura-2	> Ca^{2+} release	37
PS1-L286V	PC12 cells	Fura-2	> Ca^{2+} release	38
Sporadic AD/ageing	T lymphocytes	Fura-2	> Ca^{2+} release	13
PS1-M146V	Neurons from KI mice	Fura-2	> Ca^{2+} increase	23
PS1 wt, PS1-M146V	*Xenopus* oocytes	Oregon Green-5N	> Ca^{2+} release	44
PS1-L286V	Ntera-2 cells	Fura-2	> Ca^{2+} release	74
PS2 WT, PS2-N141I, PS2-M239V	*Xenopus* oocytes	Oregon green	> Ca^{2+} release	51
PS2 wt	SH-SY5Y cells	immunoprecipitation	Interaction with sorcin	79
PS1-L286V, PS1-M146V	PC12 cells, cortical neurons from KI mice	Fura-2	> Ca^{2+} release	39
PS1-M146L/V	Hippocampal slices from Tg mice	Electrophysiology	> Ca^{2+} response (depolarization)	45
PS1-M146V	Fibroblasts from KI mice	Fura-2	> Ca^{2+} release; < CCE	24
PS1-M146V	*Xenopus* oocytes	Oregon Green	> Ca^{2+} release	81
PS1-M146L, PS2-N141I	Stable SH-SY5Y clones	Fura-2	< CCE	56
PS1-A246E, PS2-N141I	Neurons from Tg mice	Fura-2	> Ca^{2+} release	40
PS1-M146V	*Xenopus* oocytes	Oregon Green	> Ca^{2+} release; > ER Ca^{2+}	55
PS1-ΔE9	SH-SY5Y cells	Fluo-3	> Ca^{2+} release	83
PS1-ΔE9	SH-SY5Y cells	Fura-2	> Ca^{2+} release; < CCE	57
PS1-A246E	Primary hippocampal neurons and slices from Tg mice	Fura-2, patch clamp	< CCE; > ER Ca^{2+}	58

Table 1 continued..

Effects of presenilins on Ca^{2+} homeostasis (II).

Presenilin mutations	**Cell models**	**Methods**	**Effects**	**Ref.**
PS1-M146V	Oligodendrocytes from KI mice	Fura-2	> Ca^{2+} release	41
PS1-KO, PS1-A246E	Hippocampal slices	Electrophysiology	> ER Ca^{2+}	59
PS1-M146V	Neurons from KI mice	Patch clamp	> Ca^{2+} release	42
PS1-KO, PS1 wt	Neurons from PS1-KO neurons	Fura-2	< Ca^{2+} release	63
PS2-M239I	FAD-fibroblasts HeLa cells, HEK293	Fura-2, aequorin	< Ca^{2+} relase; = CCE	75
PS2-N141I, PS2-M239V, PS2-D263A	HEK 293	Fura-2	> CCE (PS2-D263A)	76
PS2-T122R	FAD-fibroblasts HeLa cells, HEK293	Fura-2, aequorin	< Ca^{2+} relase; < CCE	22
PS2-KO	Primary neurons	Patch clamp	> VGCC currents	64
PS1-M146V (+ Tau-P301L + APPswe)	Primary cortical neurons from 3xTg mice and KI mice	Fura-2	> Ca^{2+} release	52
PS2-KO	Left ventricular papillary muscles	Aequorin	> Ca^{2+} relase	62
PS2-N141I	Primary cortical neurons from KI mice	Fura-2	> Ca^{2+} release	47
PS2-N141I	PC12 cells, primary cortical neurons from KI mice	Fura-2	> Ca^{2+} release	48
PS2 wt (loop peptide)	NRP-154 cells	Fura-2	< ER Ca^{2+}	87
PS1-P117L, PS1-M146L, PS1-L286V, PS1-A246E, PS2-T122R, PS2-M239I, PS2-N141I, PS2-D366A	SH-SY5Y cells, HeLa cells, HEK293, wt and DKO MEFs, primary neurons	Aequorin, Fura-2	< ER Ca^{2+}; < Golgi Ca^{2+}	67
PS-DKO	wt and DKO MEFs	Aequorin	< ER Ca^{2+} in DKO	49
PS1wt, PS1-M146V, PS1-DE9, PS1-D257A, PS2-N141I	Sf9 cells microsomes, wt and DKO MEFs	Lipidic bilayers, Fura-2, Mag-Fura-2	> ER Ca^{2+}; > Ca^{2+} release	69
PS1-M146V (+ Tau-P301L + APPswe)	Cortical slices from 3xTg mice and KI mice	Patch clamp, two photons calcium imaging	> Ca^{2+} release; > CICR	43
PS1 wt (NTF)	Cerebellar microsomes	Patch clamp	> RyR gating	53
PS-DKO	PS1/PS2 DKO lymphocytes	Fluo-3	< Ca^{2+} relase; < ER Ca^{2+}	65

Table 1 continued..

Effects of presenilins on Ca^{2+} homeostasis (III).

Presenilin mutations	Cell models	Methods	Effects	Ref.
PS2 wt (NTF)	Cerebellar microsomes	Patch clamp	> RyR gating	54
PS1-M146V, PS2-N141I	Sf9 cells, DT40 lymphocytes	Electrophysiology, fura-2, Mag-Fura-2	> Ca^{2+} release; = ER Ca^{2+}	50
PS1-M146V, PS-KO/DKO	wt, KO and DKO MEFs, *Xenopus* oocytes	Fura-2, Oregon green	> SERCA activity; > Ca^{2+} release;	70
PS2 wt	SH-SY5Y cells	Aequorin, Fura-2	< ER Ca^{2+}	77
PS1 wt, PS1-D257A	Rat DRG neurons	Indo-1	> CCE	60
PS1 wt, PS1-S170F, PS1-I213F, PS1-E318G, PS1-P117R, PS1-L226F	wt and PS1/PS2 KO MEF, HEK 293 cells	Fura-2	> CCE (DKO); < CCE (FAD PS1)	61
PS2 wt, PS2-T122R, PS2-D366A	SH-SY5Y cells, HeLa, wt and DKO MEFs	Aequorin	< ER Ca^{2+}; < SERCA activity; > IP_3R/RyR leak	68

Exceptions to the rule: Is the Ca^{2+} overload hypothesis still tenable?

Although the ‘‘Ca^{2+} overload’’ hypothesis is frequently invoked, different studies reported either no alteration or a reduced store Ca^{2+} content in cells expressing wt or FAD-mutant PSs [22, 50, 67, 74-77]. In particular, it was demonstrated that the FAD-linked PS2 mutations M239I and T122R reduce rather than increasing Ca^{2+} release in fibroblasts from FAD patients and in cell lines stably or transiently expressing the PS2 mutants (Fig. 1A) [22, 67, 75]. In addition, an extended investigation of other FAD-linked PS mutants (PS2-N141I, PS1-A246E, PS1-L286V, PS1-M146L, PS1-P117L), by directly monitoring the ER and the Golgi Ca^{2+} content in different cell lines, confirmed that the FAD-linked PS2 mutations cause a reduction in the Ca^{2+} level of these organelles and none of the PS1 mutations cause an increase [67]. Moreover, PS1 and PS2 mutants were also tested in rat cortical neurons upon transient co-expression with green fluorescent protein (GFP): among GFP-positive cells, only those expressing PS2-T122R, but not PS1-A246E or PS1-M146L, showed a marked reduction of Ca^{2+} release induced by an IP_3 generating agonist (Fig. 1B). Importantly, a mild reduction of the store Ca^{2+} content rather than a Ca^{2+} overload was also recently found in DT40 cells expressing PS1-M146L; similarly, no Ca^{2+} overload was found at the intracellular store level, in rat primary neurons over-expressing the same PS1 mutant [50].

The absence of elevated ER [Ca^{2+}] in these models suggests that the enhanced Ca^{2+} release observed by other groups, upon expression of various FAD-linked PS mutants, might depend on different mechanisms (see below), not necessarily due to an increased amount of Ca^{2+} within the stores. Consequently, the “Ca^{2+} overload” hypothesis often takes the form of an “exaggerated Ca^{2+} release”, not necessarily reflecting the store Ca^{2+} content [78].

Notwithstanding, a dysregulation of store Ca^{2+} handling seems the common feature of a heterogeneous group of FAD-linked PS mutants. The causal linkage between the precise alteration and the development of the disease has however still to be investigated. On this line, the fact that, at variance with different FAD mutations in PS1, the PS2 mutations N141I, T122R, M239I, induce

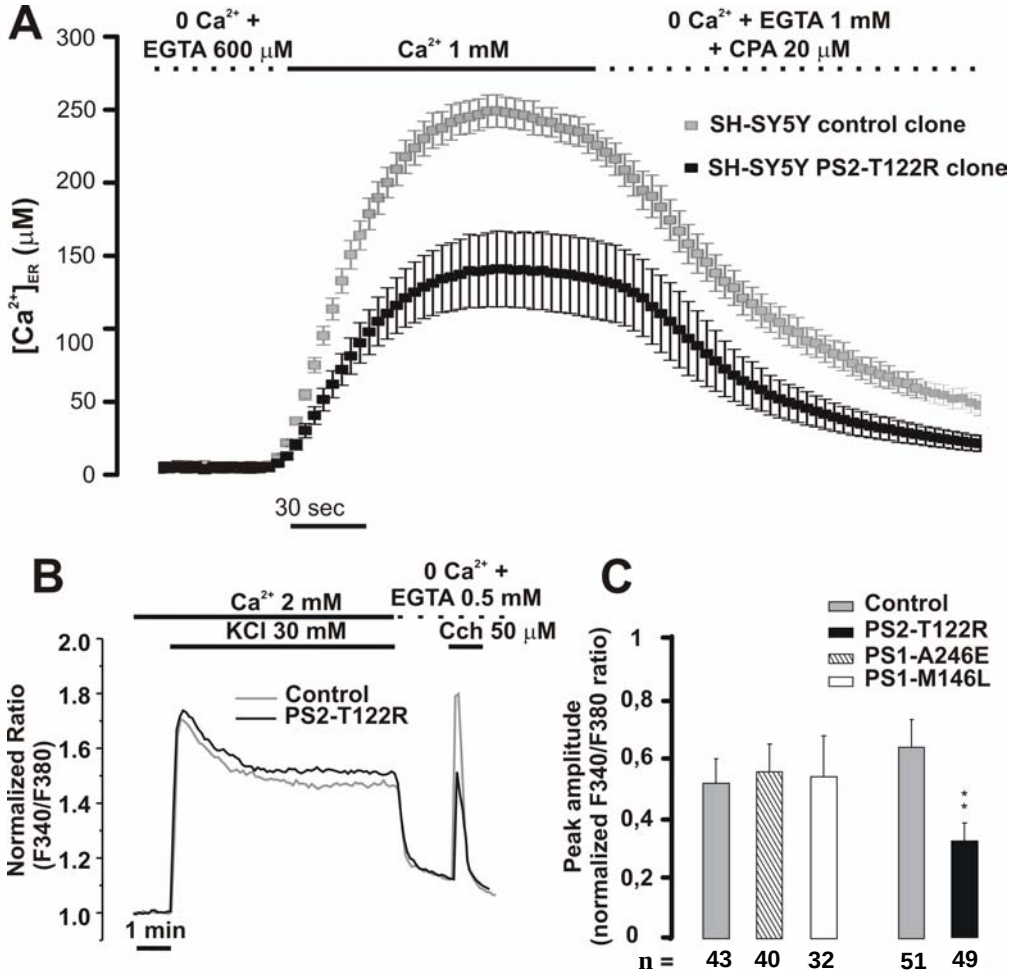

Figure 1. The FAD-linked PS2-T122R reduces the ER Ca^{2+} level.
(A) $[Ca^{2+}]_{ER}$ measurements in SH-SY5Y cells stably expressing PS2-T122R. Cell clones were transfected with the cDNAs coding for ER-aequorin. After aequorin reconstitution, control (grey trace) and PS2-T122R expressing (black trace) cells were bathed in a Ca^{2+}-free, EGTA-containing medium (600 μM). $CaCl_2$ (1 mM) was first added to follow the ER refilling process; subsequently, the cells were exposed to the SERCA inhibitor cyclopiazonic acid (CPA, 20 μM) in the Ca^{2+}-free EGTA-containing medium (1 mM), to estimate the passive ER Ca^{2+} leak (mean ± s.e.m, n=6 independent experiments). (B) Cytosolic Ca^{2+} changes measured with Fura-2 in neurons, co-transfected with the cDNA coding for GFP and PS2-T122R (black trace) or with the void-vector (grey trace). The cells were challenged in a Ca^{2+}-free, EGTA-containing medium with the IP_3-generating agonist carbachol (Cch, 50 μM) after 6 min exposure to KCl (30 mM) to load neuronal Ca^{2+} stores. Traces are averages of 3 GFP positive neurons and represent ratio (F340/F380) normalized to the value obtained within the first minute of the experiment. (C) Bars represent the peak amplitude above basal level, measured in neurons expressing different PS mutants, as shown in panel B. Values are expressed as Δratio (F340/F380), (mean ± s.e.m, n = number of GFP positive neurons, ** $p < 0.001$ unpaired Student's *t* test). Panel B was adapted from [67].

a partial ER Ca^{2+} depletion, can be envisaged as a protective event against the possible damage caused by Aβ oligomers or oxidative stress, given the anti-apoptotic effect of low ER Ca^{2+} levels [8]. Accordingly, mutations in PS2 appear to be endowed of a lower toxicity (with respect to those in PS1), considering also the later onsets and milder AD phenotypes usually associated to those mutations. The Ca^{2+} dysregulation so often described in different AD models could thus be considered as either a positive or a negative modulator, rather than a causative factor involved

in the neurodegeneration which is typical of the disease [28].

Presenilins and Ca^{2+} dysregulation: Mechanisms of action

The hypothetical mechanisms through which PS mutants exert their effect on Ca^{2+} homeostasis are numerous (Fig. 2). In addition to those mentioned above, i.e. increased expression or sensitivity of the ER Ca^{2+} release channels (RyR and IP_3R) [35, 39, 42-54] and modulation of CCE [24, 56-61], other mechanisms have been implicated, on the basis of either physical or functional interactions with proteins involved in cellular Ca^{2+} handling. In particular, it has been shown that a molecular link exists between the large hydrophilic loop region of PS2 and sorcin, a penta-EF-hand Ca^{2+}-binding protein that serves as a modulator of RyRs [62, 79], whereas it was shown that a member of the recoverin family of neuronal Ca^{2+}-binding proteins, calsenilin, upon interaction with PS1 and PS2 holoproteins, regulates the level of a PS2 proteolytic product [80] and reverses the PS-mediated enhancement of Ca^{2+} signalling [81]. The same protein was shown to interact directly, in a Ca^{2+}-independent way, with PS2 and potentiate the PS2-promoted reduction in the ER Ca^{2+} content upon co-expression in SH-SY5Y cells [77]. Also the Ca^{2+}-binding protein calbindin D28k has been involved in the PS-dependent Ca^{2+} response: its over-expression suppresses $[Ca^{2+}]_i$

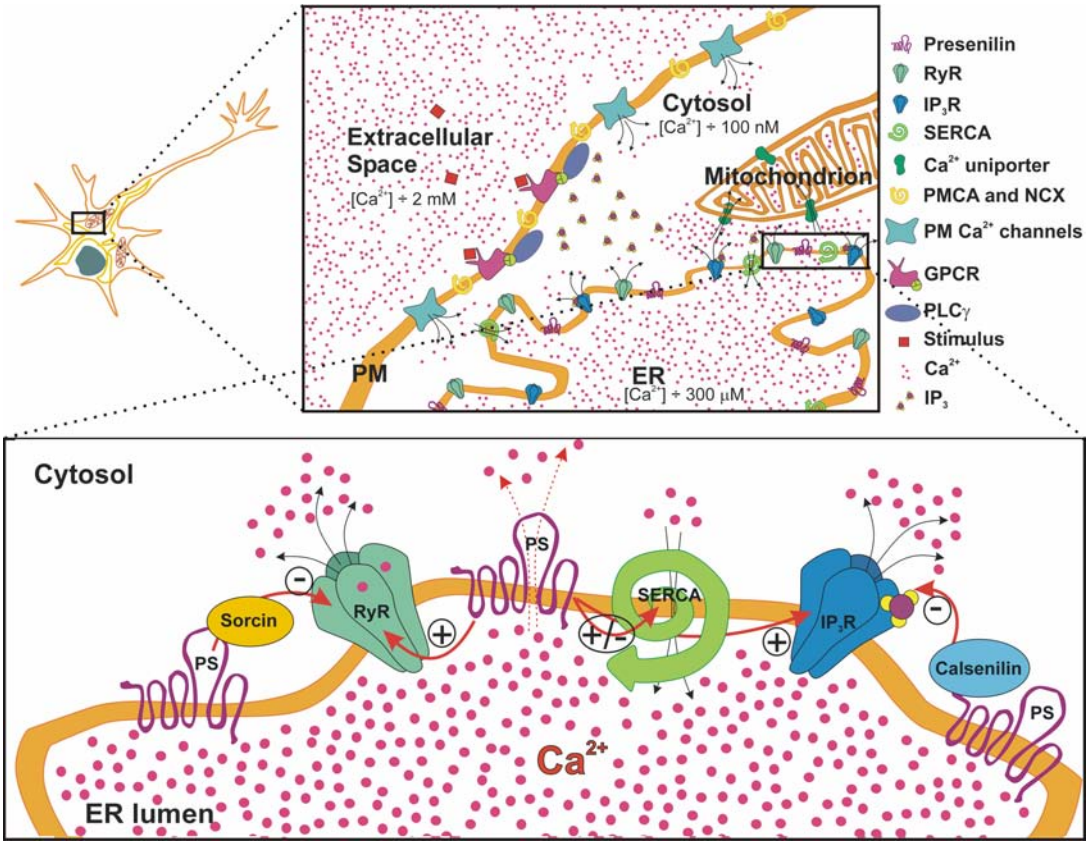

Figure 2. Model of presenilin's effects on Ca^{2+} homeostasis.
FAD-linked PS mutants alter, directly or indirectly (via sorcin or calsenilin), the expression/sensitivity of ER Ca^{2+} release channels (RyRs and IP_3Rs), leading to an exaggerated ER Ca^{2+} release that in turn may cause abnormal mitochondrial Ca^{2+} uptake. Full-length wt PSs, but not FAD mutants, form Ca^{2+} permeable leak channels across the ER membrane: an enhanced Ca^{2+} release is due to Ca^{2+} accumulation because of defective leak channels. PSs interact with the SERCA pump either reducing or increasing its activity. See text for details.

rises, generation of reactive oxygen species and apoptosis, induced by Aβ, in cultured neural cells expressing the PS1 mutations L286V or M146V [82].

As far as Ca^{2+} signalling is concerned, an enhanced phospholipase C activity has been linked to the increased Ca^{2+} release observed upon stimulation, in SH-SY5Y cells expressing PS1-ΔE9 [83]. In DT40 cells, stably expressing PS1-M146L, Cheung *et al.* reported an increased IP_3Rs' sensitivity that leads to exaggerated Ca^{2+} responses, yet in the presence of reduced ER Ca^{2+} levels [50]. The effect of FAD-linked PS mutants on IP_3R gating seems to be direct and unrelated to the known modulation of channel's activity by elevated luminal $[Ca^{2+}]$, since an enhanced Ca^{2+} release, at basal, was recorded in cells with a reduced ER Ca^{2+} content [50]. Conversely, it was shown that wt PSs form Ca^{2+} permeable channels in planar lipid bilayers [69], whereas FAD-linked PS mutants resulted in channels with reduced ionic conductance [69, 84]. A straightforward model was thus suggested in accordance with the Ca^{2+} overload hypothesis: PSs represent one of the molecular components of the elusive Ca^{2+} leak channel of the ER membrane and the FAD-linked PS mutants, by reducing the leak, increase the ER Ca^{2+} content. Further support to the Ca^{2+} overload hypothesis has recently been provided in *X. laevis* oocytes expressing PS1-M146V (and wt PS1 or 2, albeit to a lesser extent). In this experimental model, expression of the PS1 mutant increases cytosolic Ca^{2+} clearance, an effect also mimicked by SERCA-2B overexpression [70]. In contrast, by measuring Ca^{2+} accumulation inside the stores by an ER-located aequorin, it has recently been shown that the FAD mutant PS2-T122R inhibits ER Ca^{2+} rises also in permeabilized cells (Fig. 3A) [68]. Under these conditions, the ER Ca^{2+} uptake rate (Fig. 3B) simply reflects the pump activity, since it is not affected by Ca^{2+} influx/extrusion processes across the plasma membrane and by mitochondria Ca^{2+} buffering. In the same study, a modest but significant increase in Ca^{2+} leakage across the ER was reported in intact cells expressing the PS2 mutant, upon SERCA pump inhibition (Fig. 3C). The effect was attributed to Ca^{2+} leak through the RyRs and IP_3R-3 but not the translocon, the protein import machinery recently indicated as a relevant ER Ca^{2+} efflux pathway [85, 86].

In wt MEFs, the knocking down, by siRNAs, of the endogenous level of PS2, but not PS1, increases both ER Ca^{2+} pumping rates and steady-state levels, suggesting that endogenous PS2 works as a brake on ER Ca^{2+} uptake [68]. Interestingly, in the rat prostate adenocarcinoma cell line NRP-154, the caspase-3 generated loop peptide (AA 308-329) of PS2, but not of PS1, is able to reduce the ER Ca^{2+} content through a functional interaction with the IP_3R that, in turn, shows a marked activation of its gating kinetics [87]. On the same line, in neuroblastoma cells, reduction of ER Ca^{2+} levels have been obtained only upon over-expression of the wt form of PS2, but not of PS1 [77]. Altogether these findings suggest a peculiar role of PS2 in ER Ca^{2+} handling.

Is Ca^{2+} dysregulation worth of therapeutical interventions in AD? Open questions

The linkage between PSs and Ca^{2+} handling has actively been investigated in the last years, but the results are still contradictory: the possibilities of therapeutic interventions at this level are far from being on the pipeline and need to be carefully considered (see below). Notwithstanding, because of the relevance of cellular Ca^{2+} homeostasis not only in AD but also in other neurodegenerative processes, it appears vital to clarify the role played by PSs at this level. In particular, major advances are expected by addressing the following issues:

i) thoroughly investigating the role of PSs, as well as of γ-secretases, in normal aging brain and sporadic AD;
ii) identifying at which functional and structural level endogenous PS2 (and PS1) interact with the Ca^{2+} homeostatic machinery, as both full length and dimeric form, either alone or combined with other proteins in various complexes. Regarding this, the complexity of the crosstalk between PS1 and PS2 should also be considered, as recently pointed out in an elegant work by Placanica *et al.* [88]. These authors demonstrated the capability of FAD-linked PS1 mutants to shift the balance from PS1 to PS2 containing complexes. Furthermore, in the same work,

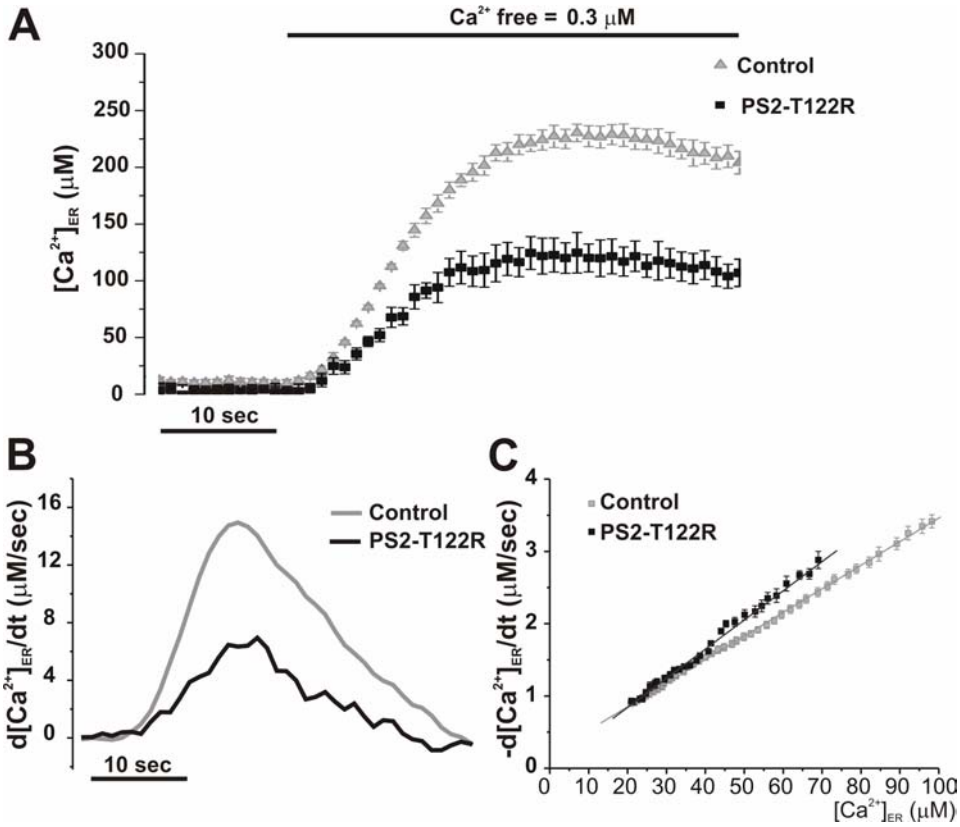

Figure 3. PS2-T122R reduces SERCA activity and increases passive ER Ca^{2+} leak
(A) SH-SY5Y cells were transiently co-transfected with the cDNAs coding for ER-aequorin and PS2-T122R (black trace) or with the void vector as control (grey trace). Upon aequorin reconstitution, the cells were washed and bathed in a Ca^{2+}-free, EGTA-containing medium (0.6 mM). The cells were then permeabilized for 2 minutes with digitonin (20 μM) in a EGTA-containing intracellular solution. After digitonin washing, ER Ca^{2+} uptake was followed in the same solution containing an EGTA-based Ca^{2+} buffer (free $[Ca^{2+}]$=0.3 μM) and ATP (0.2 mM). Traces were aligned to Ca^{2+} addition (mean ± s.e.m., n=12). (B) The rate of ER Ca^{2+} uptake was estimated from the first derivative of ER $[Ca^{2+}]$ rises measured upon Ca^{2+} addition in permeabilized cells as shown in panel A. (C) The passive ER Ca^{2+} leak ($-d[Ca^{2+}]/dt$) was estimated in intact cells by ER-aequorin upon CPA addition, as shown in Figure 1A. Values were plotted as a function of the instantaneous $[Ca^{2+}]_{ER}$, estimated from the single traces (grey and black symbols, for control and PS2-T122R expressing cells, respectively; mean ± s.e.m, n=24). (Panel C was adapted from [68] with permission from Blackwell publishing Ltd).

they also suggested that this type of analysis is not so straightforward. In fact, γ-secretase activity does not always correlate with total expressed subunit levels since only a fraction of expressed subunits, as measured by western blotting, are actually incorporated into enzymatically active complexes;

iii) defining for each novel target/function the precise effect of FAD-linked mutations, i.e. do they represent a loss- or a gain-of-function? Are DKO cells always suitable models to answer these questions?

iv) distinguishing between Ca^{2+} defects that are a consequence of PS-dependent Aβ production - being likely late events - from Ca^{2+} defects that can unambiguously attributable to PS themselves, either devoid or endowed of enzymatic activity. These latter in fact are expected to play a major role in the initial phase of the disease and might thus represent a precocious drug target;

v) precisely defining when, where and which type of PS-dependent Ca^{2+} dysregulation

takes place at the level of CNS. Aside from neurons, which are undoubtedly the primary site of memory deficit, also astrocytes are likely involved in memory loss and brain damage. Considering that in astrocytes intracellular stores and CCE are primary sources of Ca^{2+} changes, it is conceivable that endogenous and mutant PSs likely play specific early effects also at this level, an issue which has not yet received enough consideration but looks extremely promising [89]. Contradictory data on PS-dependent Ca^{2+} dysregulation might be solved also by expanding the type of Ca^{2+} analysis from cytosol to inside organelles (ER, mitochondria, Golgi and vesicles) by means of fluorescent ratiometric Ca^{2+} probes, selectively targeted to these locations and suitable to single cell measurements, both *in vitro* and *in vivo* [90].

Finally, by dealing with this issue, attention should also be paid to the employed terminology. The term "Ca^{2+} overload" should be restricted to indicate Ca^{2+} loads inside the stores whereas the term "Ca^{2+} hyperactivity" should better be referred to increased cytosolic Ca^{2+} levels. Interestingly, in Ca^{2+} imaging studies, carried out by means of cytosolic Ca^{2+} probes in different Tg AD mouse models, based on combined mutations in APP and/or PS, a neuronal Ca^{2+} hyperactivity was found only at late stages. This latter was dependent on Ca^{2+} entry across the plasma membrane and showed a close proximity to plaque deposition [91]. Conversely, in similar Tg mouse models, a "Ca^{2+} overload" at the store level was found already in neurons from newborn mice [52, 92].

Within the frame of the "Ca^{2+} hypothesis" for AD pathogenesis, therapeutic interventions have been suggested that are specifically aimed at reducing the Ca^{2+} hyperactivity by means of L-type Ca^{2+} channel and NMDA receptor antagonists [7]. However, it should be mentioned that the classical Ca^{2+} antogonists nimodipine and nitrendipine, as well as the known CCE inhibitors clotrimazole and SKF96365 were reported to increase Aβ42 secretion in a way independent of Ca^{2+} entry and without affecting the total Aβ levels [93]. Conversely, in the total absence of data concerning the Ca^{2+} dynamics and steady-state levels of intracellular Ca^{2+} stores in brain cells, from both normal and AD mouse models, pharmacological targeting of Ca^{2+} release channels or hypothetical leak pathways sounds still premature.

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