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Reduction of Ca^{2+} stores and capacitative Ca^{2+} entry is associated with the familial Alzheimer's disease presenilin-2 T122R mutation and anticipates the onset of dementia

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Mutations in the presenilin genes *PS1* and *PS2*, the major cause of familial Alzheimer's disease (FAD), are associated with alterations in Ca^{2+} signalling. In contrast to the majority of FAD-linked PS1 mutations, which cause an overload of intracellular Ca^{2+} pools, the FAD-linked PS2 mutation M239I reduces Ca^{2+} release from intracellular stores [Zatti, G., Ghidoni, R., Barbiero, L., Binetti, G., Pozzan, T., Fasolato, C., Pizzo, P., 2004. The presenilin 2 M239I mutation associated with Familial Alzheimer's Disease reduces Ca^{2+} release from intracellular stores. Neurobiol. Dis. 15/2, 269–278]. We here show that in human FAD fibroblasts another PS2 mutation (T122R) reduces both Ca^{2+} release and capacitative Ca^{2+} entry. The observation, done in two monozygotic twins, is of note since only one of the subjects showed overt signs of disease at the time of biopsy whereas the other one developed the disease 3 years later. This finding indicates that Ca^{2+} dysregulation anticipates the onset of dementia. A similar Ca^{2+} alteration occurred in HeLa and HEK293 cells transiently expressing PS2-T122R. Based on these data, the "Ca^{2+} overload" hypothesis in AD pathogenesis is here discussed and reformulated.

Keywords: Presenilin; Alzheimer's disease; Ca^{2+} homeostasis; Capacitative Ca^{2+} entry; Monozygotic twins; Fibroblasts

Introduction

Genetic factors are involved in the aetiology of dementia. Three genes have been identified which, when mutated, cause familial Alzheimer's disease (FAD): the presenilin-1 (*PS1*), the presenilin-2 (*PS2*) and the amyloid precursor protein (*APP*) genes (Cruts et al., 1998; Finckh et al., 2000a; Goate et al., 1991; Sherrington et al., 1995).

As far as PS is concerned, the two genes (and proteins) show high homology although genetic and physiological differences between the two molecules have been described (Lai et al., 2003). Over 130 mutations have been identified in *PS1* but, so far, only 10 mutations in *PS2* have definitively been linked to FAD (Binetti et al., 2003; Ezquerra et al., 2003; Finckh et al., 2000a; Levy-Lahad et al., 1995; Rogaev et al., 1995; Tedde et al., 2003). In general, mutations in *PS2*, if compared to *PS1*, result in a relatively mild clinical phenotype that is characterised by a wider range of disease onset (from 45 to 88 years), with prominent features similar to sporadic AD (Finckh et al., 2000b; Sherrington et al., 1996).

Identification of these genes and associated mutations was extremely important for the understanding of the AD aetiology. The majority of APP and PS mutations result in increased production/aggregation of amyloid β-peptide (Aβ), the main constituent of senile plaques. Although the mechanism of neuronal damage by Aβ is uncertain, the amount of protofibrillar Aβ correlates with oxidative damage to lipids, proteins and nucleic acids in the brain of patients, resulting in impaired synaptic plasticity and neuronal degeneration (Takeda et al., 2004). PS mutations also result in major defects in Ca^{2+} handling by the endoplasmic reticulum (ER), which may perturb synaptic function while contributing to neurodegeneration. Studies in transgenic mice that express mutant forms of APP and/or PS provided evidence of altered neuronal Ca^{2+} homeostasis and synaptic plasticity with impaired learning and memory (Hsia et al., 1999; Hsiao et al., 1996; Schneider et al., 2001). Thus, besides the abnormal processing of APP, perturbed

Abbreviations: FAD, familial form of Alzheimer's disease; PS, presenilin; APP, amyloid precursor protein; Aβ, amyloid β-peptide; ER, endoplasmic reticulum; CCE, capacitative Ca^{2+} entry; AD, Alzheimer's disease; FHC, familial healthy controls; HC, healthy controls; mKRB, modified Krebs–Ringer buffer; AEQ, aequorin; dox, doxycycline; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; CPA, cyclopiazonic acid; $InsP_3$, inositol 1,4,5-trisphosphate; Hist, histamine; Cch, carbachol.

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intracellular Ca^{2+} homeostasis is also considered a common feature of FAD-linked PS mutations. The nature of this Ca^{2+} dysregulation is, however, still controversial.

We previously showed that the FAD-associated PS2 mutation M239I reduces Ca^{2+} release from intracellular stores (Zatti et al., 2004), a phenotype just opposite to that reported for the large majority of PS1 mutations (Chan et al., 2000; Guo et al., 1996, 1997; Herms et al., 2003; Leissring et al., 2000a,b; Popescu et al., 2004; Schneider et al., 2001; Smith et al., 2002; Stutzmann et al., 2004). We wondered whether this rather surprising effect could be common to other FAD-linked PS2 mutations, thus helping to explain the typical features of their associated forms of dementia (Finckh et al., 2000b; Sherrington et al., 1996). We have therefore investigated the effect on intracellular Ca^{2+} stores and capacitative Ca^{2+} entry (CCE), the Ca^{2+} influx pathway activated by store depletion, of another PS2 mutation (T122R) that was recently described by our group (Binetti et al., 2003). Different and integrated approaches have here been employed: first, we analysed human fibroblasts obtained from subjects belonging to the PS2-T122R pedigree. We had the rather uncommon opportunity to study two monozygotic twins carrying this mutation: interestingly, at the time of fibroblasts collection, only one of them presented overt signs of disease, whereas the other one was declared ill only 3 years later. Second, the effect on Ca^{2+} handling of the mutation was evaluated in two model cell lines (HeLa and HEK293) transiently transfected with wt- and mutant-*PS2*. Third, by using in HeLa cells an inducible expression system, we studied the relationship between the expression level of mutant PS2 and the entity of Ca^{2+} dysregulation.

We here show evidence that PS2-T122R consistently reduces the ER Ca^{2+} content and induces, in addition, a substantial CCE inhibition. Remarkably, we also found that Ca^{2+} responses of FAD-primary fibroblasts from the two monozygotic twins were similarly affected in spite of their different phenotype, suggesting that Ca^{2+} dysregulation due to PS2-T122R is an early event in AD and anticipates the onset of dementia.

The finding that, contrary to PS1, two PS2 mutations, M239I and T122R, reduce the Ca^{2+} content of intracellular stores, forces us to reconsider the general hypothesis of the "ER Ca^{2+} overload" in FAD and rises the relevant question of the role played by Ca^{2+} dysregulation in the different forms of the disease.

Materials and methods

Subjects

Patients included in this study were recruited and evaluated at the Memory Clinic of IRCCS "Centro San Giovanni di Dio-Fatebenefratelli", Brescia, and at Neuroscience Department AFaR Fatebenefratelli Hospital, Rome, Italy. Written informed consent was obtained from all subjects or, where appropriate, their caregivers, following the procedures approved by the hospital local ethical committee. Full description of the Italian pedigree with FAD-associated PS2 T122R mutation was reported elsewhere (Binetti et al., 2003). We obtained fibroblast cell lines from monozygotic twins (subjects III-2 and III-3, see Table 1) carrying the PS2 T122R mutation and from two healthy controls, related with the family (FHC), not carrying the mutation (subject FHC-1, previously indicated as III-4, and subject FHC-2, brother and cousin of the twins, respectively; see Table 1). At the time of fibroblast collection, subject III-2 was affected, while subject III-3 was still unaffected. Additionally, we included in the present study unrelated healthy controls (HC, see Table 1). Global cognition was investigated by Mini-Mental State Examination (MMSE) as previously described (Folstein et al., 1975). Demographic and clinical features of subjects are reported in Table 1.

Human primary skin fibroblast cultures

Fibroblast primary cultures were derived from the dermal biopsy of FAD PS2-T122R and FHC subjects as previously described (Govoni et al., 1993). Fibroblasts from unrelated healthy controls (HC) were obtained from Fatebenefratelli Biological Repository (F-BR) of IRCCS "Centro San Giovanni di Dio-Fatebenefratelli", Brescia, Italy. Cells were cultured at 37°C in 5% CO_2/95% air in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, San Diego, CA, USA), supplemented with 10% foetal

Table 1
Clinical and demographic characteristics

Subjects	PS2 T122R mutation	Age (years)	Gender	Diagnosis	Duration of disease (years)	MMSE
PS2 T122R pedigree						
III-2 (twin)	+	60	F	FAD	3	15/30
III-3 (twin)	+	60	F	No disease	–	30/30
FHC-1/III-4 (brother)	–	57	M	No disease	–	27/30
FHC-2 (cousin)	–	57	F	No disease	–	29/30
Healthy controls (HC)						
Mean values ± SD		67.5 ± 4.0				28.25 ± 1.6
HC-1		65	F	No disease	–	30/30
HC-2		70	F	No disease	–	28/30
HC-3		70	M	No disease	–	30/30
HC-4		65	M	No disease	–	30/30
HC-5		69	F	No disease	–	27/30
HC-6		66	M	No disease	–	27/30
HC-7		74	M	No disease	–	28/30
HC-8		61	F	No disease	–	26/30

MMSE = Mini-Mental State Examination; F = female, M = male.

calf serum (FCS, Gibco, Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and with non-essential amino acids. The different fibroblast cell cultures were plated, at the same passage number, on cover slips (BDH, Milan, Italy) and used, after 2 days, for Ca^{2+} measurements.

Ca^{2+} measurements

Cells, plated on cover slips (24 mm diameter), were loaded with fura-2 by incubation with 2 μM fura-2/AM at room temperature for about 60 min in MEM containing 10% FCS and 0.04% pluronic. To prevent fura-2 leakage and sequestration, 250 μM sulfinpyrazone was present throughout the loading procedure and $[Ca^{2+}]_i$ measurements. The cover slips were washed with 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, at 37°C), mounted on a thermostated chamber (Medical System Corp., NY, USA), placed on the stage of an inverted microscope (Zeiss, Axiovert 100 TV) equipped for single cell fluorescence measurements and imaging analysis (TILL Photonics). The sample was alternatively illuminated (t = 10 ms) by monochromatic light (at 340 and 380 nm) every second through a 40× oil immersion objective (NA = 1.30; Zeiss). The emitted fluorescence was passed through a dichroic beamsplitter (455DRPL), filtered at 505–530 nm (Omega Optical and Chroma Technologies, Brattleboro, VT, USA) and captured by a cooled CCD camera (Imago, TILL Photonics, Martinsried, Germany). Cells were challenged with different stimuli by either perfusion or quick mixing at 37°C. For presentation, the ratios (F340/F380) were off-line averaged (20–30 cells) and normalised to the resting value measured within the first minute of the experiment. Traces are representative of at least 10 independent experiments. Where indicated, in the mKRB $CaCl_2$ was omitted and EGTA (150 μM) was added (Ca^{2+}-free medium), or NaCl was substituted by 140 mM KCl (K^+-based medium).

Plasmids construction

The pcDNA3 construct containing the cDNA encoding for PS2-T122R was created by site-directed mutagenesis of pcDNA3 PS2-wt (QuikChange Site-directed mutagenesis Kit, Stratagene, La Jolla, CA). The pMAR Responder vector was obtained by removing the transactivator coding region from the pKBMΔpΔMCR plasmid, kindly provided by Dr. RJ Wall (Wells et al., 1999). Full-length PS2-wt cDNA was subcloned into the *Pme*I site of pMAR plasmid (pMAR PS2-wt). The pMAR PS2-T122R was created by site-directed mutagenesis of pMAR PS2-wt. The constructs were checked by sequence (ABI Prism Genetic Analyzer 310, Applied Biosystems, Monza, Italy).

Cell lines and transfection

Human embryonic kidney (HEK) 293 cell lines were grown in F12/DMEM supplemented with 10% FCS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Transient transfections of HEK293 cells were performed by the Fugene methodology (Roche, Molecular Biochemicals, Indianapolis, IN, USA) employing 0.3 μg of aequorin (AEQ) cDNA, together with 1.2 μg of pcDNA3 vector or pcDNA3 construct containing the cDNA encoding either for human PS2-wt or PS2-T122R mutation. After 36 h, cells were used for intracellular Ca^{2+}-AEQ measurements (see below).

HeLa cells were grown in DMEM supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (100 μg/ml). Before transfection, cells were seeded onto 13-mm glass cover slips and allowed to grow to 50% confluence. At this stage, transfections were carried out using the Ca^{2+}-phosphate technique in the presence of 4 μg of DNA [3 μg PS2 (wt or T122R) cDNA or void vector (pcDNA3; Invitrogen, Milan, Italy) + 1 μg AEQ cDNA]. Intracellular Ca^{2+} measurements were carried out 48 h after transfection by means of the AEQ technique as previously described (Brini et al., 1995) and here briefly summarised.

Aequorin measurements

Cells, seeded on cover slips, were incubated with 5 μM coelenterazine for 1–2 h in mKRB and then transferred to the perfusion chamber. All the AEQ measurements were carried out in mKRB at 37°C. Agonists and other drugs were added to the same medium, as specified in the figure legends. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) to discharging the remaining unused AEQ pool. The light signal was collected by a low-noise photomultiplier with a built-in amplifier–discriminator (Thorn-EMI photon counting board), stored on an IBM-compatible computer and off-line calibrated into $[Ca^{2+}]$ values, as previously described (Brini et al., 1995).

Inducible PS2-wt, -T122R expression system

The transactivator (t-TA)-expressing HeLa Tet-Off cell line (BD Biosciences, Clontech) was transiently transfected using the Fugene methodology in the presence of 1.5 μg of DNA [1.2 μg pMAR PS2 (wt or T122R) or pMAR void vector + 0.3 μg AEQ cDNA]. In order to modulate PS2 protein expression, increasing doses (0, 50, 125, 150, 175 pg/ml) of doxycyline (dox, Sigma, St. Louis, MO) were added to the cell medium and left until cell analysis. Western blotting and AEQ-based intracellular Ca^{2+} measurements were carried out 48 h after transfection.

Protein extracts preparation and Western blot analysis

Human primary fibroblasts, HeLa and HEK293 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in ice-cold RIPA modified lysis buffer [25 mM Tris–HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.5% sodium salt deoxycholic (NaDOC), 1% nonidet-P40 (NP-40), 0.1% SDS, 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 20 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM dithiothreitol (DTT)]. The homogenates were incubated 30 min on ice and centrifuged at 60,000 × g for 20 min at 4°C. Loading of the samples was normalised for the total content of cellular proteins determined by the BCA assay (Pierce, Rockford, IL, USA). Samples were run on a 10–20% Tris–tricine gels (Novex, Invitrogen) and then blotted onto PVDF membrane (Perkin Elmer Life Sciences, Boston, MA, USA). Immunodetection of PS2 and PS1 proteins was carried out with the polyclonal antibodies Ab-2 PC235 (Oncogene, Merck, Darmstadt, Germany) and Anti-αPS1-loop (Calbiochem, EMB Biosciences, Inc., San Diego, CA), respectively. Detection was carried out by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h. The proteins were visualised by the chemiluminescence reagent CDP-star (Perkin Elmer Life Sciences). Densitometric analyses

were performed by using NIH Image software. Means of densitometric measurements of three independent experiments, normalised by the void vector values, were compared by Student's *t* test.

Results

Primary fibroblasts from subjects carrying the PS2-T122R mutation show reduction of both Ca^{2+} release from intracellular stores and CCE

We previously demonstrated that the FAD-linked PS2 mutation M239I causes a reduction in ER Ca^{2+} content (Zatti et al., 2004). In order to verify whether this reduction is a phenotype common to other PS2 mutants, we carried out a thorough study with the new FAD-linked PS2 mutation, T122R, recently described by our group (Binetti et al., 2003).

Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored with the Ca^{2+} indicator fura-2 (see Materials and methods) in fibroblasts from two subjects carrying the PS2-T122R mutation and compared with those obtained from healthy, age-matched controls, both family related (FHC, $n = 2$) and unrelated subjects (HC, $n = 8$; see Table 1). The effects of PS2-T122R were studied at the level of both Ca^{2+} content of intracellular stores and CCE, the main Ca^{2+} influx pathway activated by store depletion (Putney, 2000). The two components, Ca^{2+} release and Ca^{2+} influx, were separately analysed by applying a previously described protocol (Zatti et al., 2004) that is here briefly summarised and shown in Fig. 1A. Store depletion was induced by adding the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 20 μM) in a Ca^{2+}-free medium; the SERCA inhibitor, by inducing the passive release of stored Ca^{2+}, caused a transient increase in $[Ca^{2+}]_i$. Addition of $CaCl_2$ (Ca^{2+}, 1 mM), in the continuous presence of CPA, induced a second, large $[Ca^{2+}]_i$ rise, due to activated CCE across the plasma membrane. Fig. 1A shows $[Ca^{2+}]_i$ changes in two representative experiments carried out with control (HC, dashed trace) and PS2-T122R (continuous trace) fibroblasts. The transient increase in $[Ca^{2+}]_i$ elicited by CPA was significantly reduced in cells carrying the PS2 mutant compared to controls. The average peak height expressed as normalised ratio above basal was 0.27 ± 0.01 (mean ΔR ± SEM, $n = 54$ independent experiments) in the former group and 0.35 ± 0.02 (mean ΔR ± SEM, $n = 71$) in the latter. Thus, the peak in $[Ca^{2+}]_i$ induced by discharging intracellular Ca^{2+} stores was diminished by about 25% in cells expressing PS2-T122R. When the integral of the $[Ca^{2+}]_i$ rise caused by CPA addition was taken as an indirect measurement of the total Ca^{2+} content of intracellular stores, a similar and significant reduction was again found in PS2-T122R fibroblasts

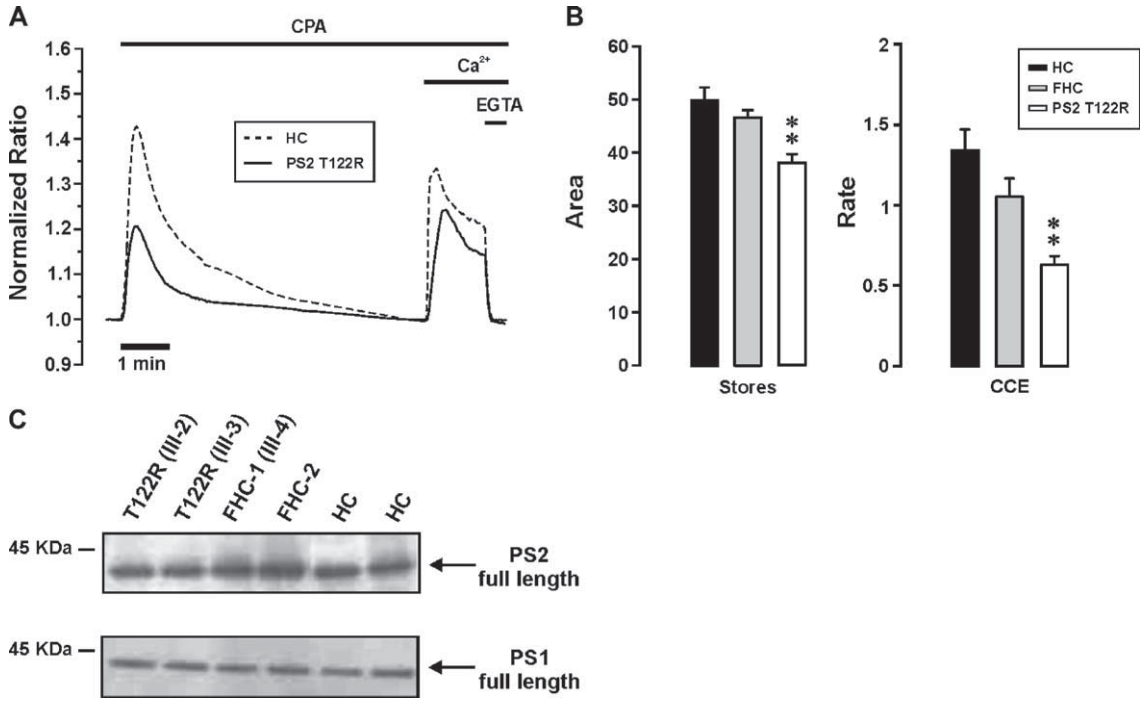


Fig. 1. Fibroblasts from subjects carrying the FAD-linked PS2-T122R mutation show reduction in both Ca^{2+} release from intracellular stores and CCE. Panel A shows a typical experiment performed with fibroblasts from PS2-T122R (continuous trace) and healthy control (HC, dashed trace) subjects. Cells, loaded with fura-2 as described in Materials and methods, were perfused in mKRB in the absence of extracellular $CaCl_2$ and in the presence of 150 μM EGTA (Ca^{2+}-free medium). CPA (20 μM), $CaCl_2$ (1 mM) and EGTA (2 mM) were added when indicated by top bars. The ratio (F340/F380) was normalised to the averaged value obtained within the first minute of the experiment. Panel B (left) shows the average Ca^{2+} content of intracellular stores, expressed as the integral of the normalised ratio trace measured above the resting value upon CPA addition (Area, mean ± SEM). Panel B (right) shows the CCE activation rate estimated upon $CaCl_2$ addition (mean ΔR/s ± SEM; n = number of independent experiments: $n = 60$ HC, $n = 40$ FHC, $n = 50$ PS2-T122R; ** = $P < 0.001$, Student's *t*-test). Panel C shows Western blot analysis of primary fibroblasts from PS2-T122R subjects (III-2 and III-3), familiar healthy controls (FHC-1 and FHC-2) and healthy controls not related with the family (HC). Cell lysates (15 μg per lane) were loaded on 10–20% denaturing Tris–Tricine gel and immunoblotted by using the polyclonal antibodies Ab-2 PC235 (Oncogene) and Anti-αPS1-Loop (Calbiochem) for PS2 and PS1 protein detection, respectively.

compared to both family unrelated (HC) and related (FHC) control fibroblasts (38 ± 2 in PS2-T122R, 50 ± 3 in HC and 47 ± 2 in FHC cells, mean ± SEM, $P < 0.001$; Fig. 1B).

In fibroblasts carrying the T122R mutation, also the Ca^{2+} influx pathway activated by store depletion showed a significant reduction, compared to controls, when measured both as peak height reached upon $CaCl_2$ addition (0.28 ± 0.02, $n = 46$, in PS2-T122R cells, continuous trace; and 0.34 ± 0.02, $n = 56$, in HC cells, dashed trace; mean ΔR ± SEM, $P < 0.001$) and as influx rate (0.6 ± 0.05 in PS2-T122R and 1.34 ± 0.1 in HC cells; mean ΔR/s ± SEM, $P < 0.001$; see Figs. 1A,B). A similar and significant reduction in CCE activation was evident also when T122R fibroblasts were compared to FHC cells (0.6 ± 0.05, $n = 46$, in PS2-T122R cells and 1.05 ± 0.1, $n = 38$, in FHC cells; mean ΔR/s ± SEM, $P < 0.001$; Fig. 1B). Since membrane potential affects the rate and extent of CCE by altering the driving force for Ca^{2+} entry, the cells were bathed in a potassium-based, depolarising medium (K^+-based medium), in order to reduce differences in this parameter among the three cell groups. Under this condition, i.e., with a collapsed membrane potential, a similar reduction in CCE was again observed upon addition of 10 mM $CaCl_2$ (instead of the standard 1 mM concentration) to obtain a more robust Ca^{2+} influx under a reduced electrical gradient (data not shown).

The alterations in cellular Ca^{2+} responses found in PS2-T122R fibroblasts could also be due to compensatory changes in PS levels. We thus carried out Western blot analysis using specific antibodies against PS1 and PS2 proteins. Fig. 1C shows that comparable levels of PS2 and PS1 were found in fibroblasts from both control and PS2-T122R subjects. This finding indicates that the altered Ca^{2+} responses observed in PS2-T122R fibroblasts were not caused by alteration in the PS expression level.

The reduction in cellular Ca^{2+} responses is an early event in the FAD-linked PS2-T122R phenotype

There are substantial evidence that alterations in Ca^{2+} signalling contribute to the pathogenesis of AD as well as of other neurodegenerative disorders (Mattson et al., 2000). How and where this event is located in the pathogenic pathway leading to FAD neurodegeneration is, however, still mysterious and no data is available yet on the relationship between the entity of this dysregulation and the severity of Alzheimer's pathology. To better address this issue, we took advantage of an unusual T122R pedigree: two monozygotic twins with only one of the two subjects (III-2) presenting overt signs of dementia at the time of investigation (see Table 1; Binetti et al., 2003).

The Ca^{2+} responses of primary fibroblasts from the two monozygotic twins carrying the PS2-T122R mutation were separately analysed. As shown in Fig. 2, the Ca^{2+} content of intracellular stores (expressed as the integral of the $[Ca^{2+}]_i$ rise caused by CPA addition) and the Ca^{2+} influx rate showed a similar level of reduction in the two sisters (about 25% and 50% reduction in store content and influx rate, respectively, compared to HC), suggesting that Ca^{2+} dysregulation occurs rather precociously and anticipates the onset of dementia. Conversely, the two analysed FHC subjects (see Table 1) showed mean values of Ca^{2+} release from intracellular stores and CCE comparable to those observed in HC subjects, unrelated to the family (see above and Fig. 1B), indicating a determinant role of the PS2-T122R mutation in the altered Ca^{2+} responses.

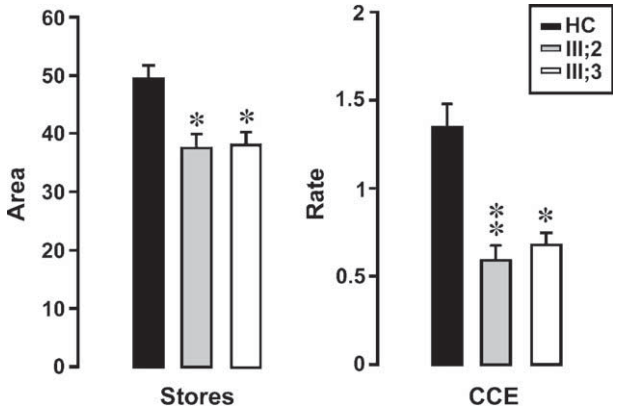


Fig. 2. Fibroblasts from the two PS2-T122R monozygotic twins show identical alterations in Ca^{2+} signalling. Cells from the two twins were treated and analysed as described in Fig. 1. The total Ca^{2+} content of intracellular stores (Area, left) and the activation rate of CCE (ΔR/s, right), estimated, respectively, upon CPA and $CaCl_2$ addition, are here reported for each subject and compared to the value obtained in HC fibroblasts (see Table 1). Means ± SEM, n = number of independent experiments: $n = 60$ HC, $n = 27$ subject III-2, $n = 23$ subject III-3; * = $P < 0.05$, ** = $P < 0.001$, Student's t test.

Overexpression of the PS2-T122R mutant is responsible for a reduced Ca^{2+} content of intracellular stores and CCE in both HeLa and HEK293 cells

To validate and strengthen the observation that PS2-T122R modifies cellular Ca^{2+} handling, we overexpressed the mutant protein in HeLa and HEK293 cells. These cell lines, at variance with primary fibroblasts, are easily transfectable with high efficiency. The technique based on the photoprotein aequorin (AEQ) as Ca^{2+} sensor (see Materials and methods) was preferred to the fura-2 technique, because first it allows cytosolic Ca^{2+} measurements at the cell population level and second it is devoid of the Ca^{2+} buffering effect, which is associated to the fura-2 approach. Transient co-expression of AEQ, with or without PS2 proteins, was preferred to the use of stable-expressing cells since this reduces variability and adaptive phenomena associated with clonal selection.

HeLa and HEK293 cells were transiently co-transfected with the cDNAs encoding for AEQ together with either human PS2-wt, PS2-T122R or the void vector (pcDNA3, see Materials and methods); PS2 overexpression was then checked by Western blot. As expected, the expression level of the exogenous proteins (both PS2-wt and T122R mutant) was much higher if compared to the level of endogenous PS2 detected in control, void vector-transfected cells (Fig. 3).

The influence of the PS2 mutation on Ca^{2+} homeostasis was then studied in these cells. Fig. 4 shows representative traces of $[Ca^{2+}]_i$ changes recorded in HeLa (panels A, B) and HEK293 (panels C, D) cells, transiently co-transfected with the void vector (control, dashed traces) or PS2 (T122R or wt, continuous traces). Maximal depletion of the ER Ca^{2+} content was obtained by stimulating the cells with CPA (20 μM) and an inositol 1,4,5-trisphosphate ($InsP_3$)—generating agonist such as histamine (Hist, 100 μM) in HeLa cells, and carbachol (Cch, 100 μM) in HEK293 cells. Compared to CPA alone, this type of stimulation induces a faster Ca^{2+} release with a sharp Ca^{2+} peak in the micromolar range that is optimally sensed by AEQ (Zatti et al., 2004). Subsequently,

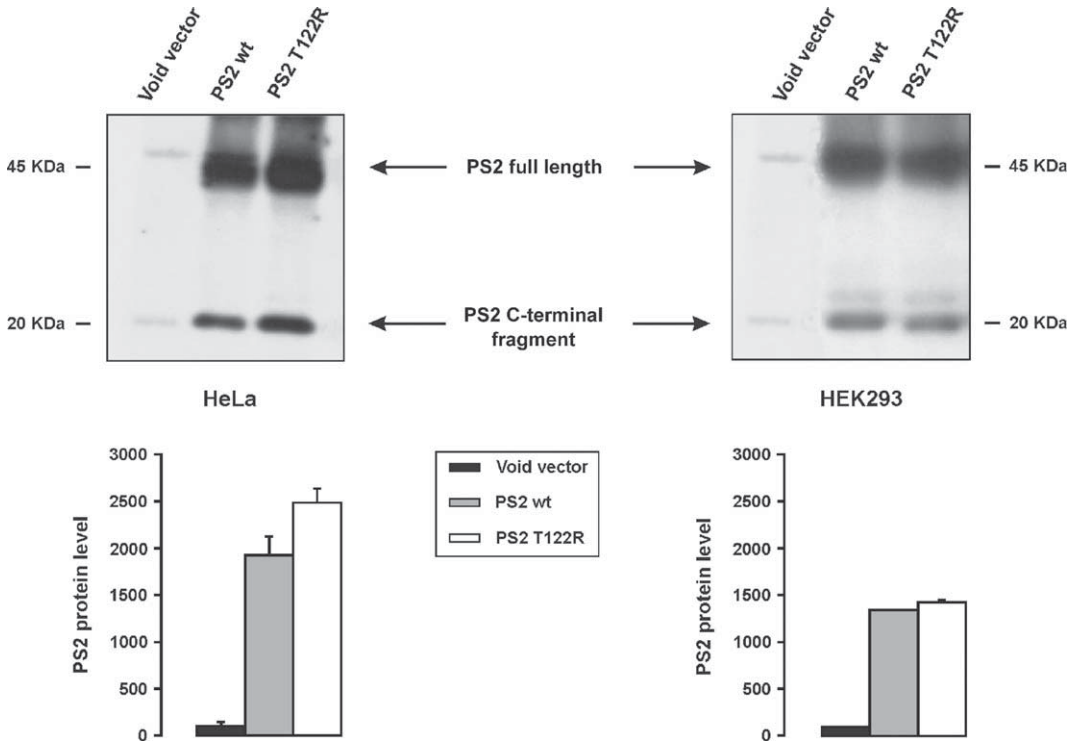


Fig. 3. HeLa and HEK239 cells transiently transfected with PS2-T122R and PS2-wt show high levels of protein expression. Western blot analysis of HeLa and HEK293 cells transiently transfected with human PS2-wt, PS2-T122R or the void vector. Cell lysates (15 μg per lane) were loaded on 10–20% denaturing Tris–Tricine gel and immunoblotted by using the polyclonal antibody Ab-2 PC235 (Oncogene) that recognises the full-length PS2 protein and the 20-kDa PS2 C-terminal proteolitic fragment. For each cell type, densitometric analysis of the bands corresponding to the full-length protein is shown in histograms (mean values ± SEM, $n = 3$).

addition of $CaCl_2$ (Ca^{2+}, 1 mM) in the continuous presence of the stimulus allowed CCE detection.

Similarly to fibroblasts from subjects carrying the PS2-T122R mutation, also the cells overexpressing the mutated protein showed a reduced Ca^{2+} release from intracellular stores compared to controls (mean peak amplitudes ± SEM of 1.56 ± 0.04 μM, for HeLa/T122R cells and 0.59 ± 0.03 μM for HEK293/T122R cells vs. 2.74 ± 0.04 and 1.03 ± 0.06 μM for respective control cells; $n = 20$ for PS2-T122R expressing cells, $n = 30$ for controls; $P < 0.001$). Thus, also in these cell models, PS2-T122R appears to be linked to a reduced Ca^{2+} release from internal stores (about 45% reduction for both cell types). By estimating the integral of the trace above the resting level, obtained upon stimulation, as an indirect measurement of the total Ca^{2+} content of intracellular stores, we found a similar entity of reduction (about 50%) in the two PS2-T122R overexpressing cell lines compared to control cells.

As far as CCE is concerned, significant differences were found between mutant PS2 overexpressing cell lines and control cells (Fig. 4). In agreement with the results obtained in PS2-T122R fibroblasts, also in HeLa and HEK293 cells overexpressing PS2-T122R, CCE was reduced by 40–45% in the average peak height reached upon $CaCl_2$ addition (0.98 ± 0.03 and 0.38 ± 0.03 μM, for HeLa and HEK293 PS2-T122R overexpressing cells, respectively; $n = 15$; 1.55 ± 0.05 and 0.7 ± 0.1 μM, for respective control cells, $n = 25$; mean peak amplitudes ± SEM, $P < 0.05$). A similar reduction was estimated by comparing the Ca^{2+} influx rate, thus reinforcing the idea that the Ca^{2+} influx pathway activated by store depletion is also affected by this PS2 mutation.

Finally, it can also be noticed that both cell types overexpressing PS2-wt (see Fig. 3) showed a reduction, compared to control cells, in both CCE and Ca^{2+} content of intracellular stores, similar to that found in PS2-T122R overexpressing cells (Fig. 4, panels B and D for HeLa and HEK293 cells, respectively). These results confirm our previous finding that overexpression of PS2-wt mimics the effect of the mutant PS2 on Ca^{2+} homeostasis (Zatti et al., 2004).

In accordance with the results obtained in primary fibroblasts, also in these cellular models, PS2 overexpression was not associated with changes in PS1 levels (data not shown).

The PS2-T122R effects on Ca^{2+} changes correlate with the protein expression level

To study in more detail the effects of PS2-T122R on Ca^{2+} homeostasis, we established a tetracycline-inducible PS2 expression system in HeLa cells. The t-TA HeLa Tet-Off cell line, stably expressing the transactivator, was transiently transfected with the pMAR void vector, pMAR PS2-wt or -T122R plasmids (see Materials and methods). The protein expression level was tested by Western blot analysis after doxycycline (dox) treatment. A gradual and proportional reduction of exogenous PS2 level was detected by increasing the dox dose (from 0 to 175 pg/ml): as shown in Fig. 5A, a complete inhibition of protein expression was reached at a drug concentration of 175 pg/ml. In fact, in this latter experimental condition, the signal detected, in PS2 (wt- or T122R) transfected cells was similar to that found in control, void vector-transfected cells, i.e., very close to the level of endogenous PS2. On the other hand, no variation in PS1 protein levels were detected in this

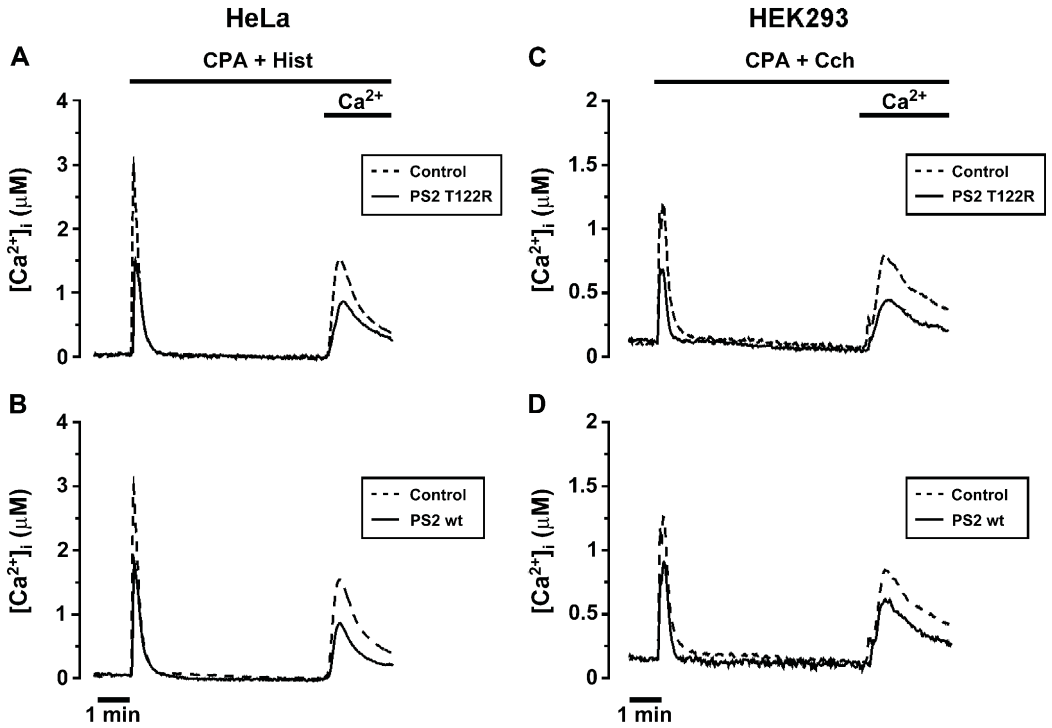


Fig. 4. HeLa and HEK293 cells transiently expressing PS2-T122R and PS2-wt exhibit similar alterations of Ca^{2+} homeostasis. HeLa and HEK293 cells were transiently transfected with the void vector (control, dashed traces) or the cDNA encoding for wt or mutant PS2 (continuous traces), in the presence of the cDNA for AEQ, as described in Materials and methods. Upon perfusion in a Ca^{2+}-free medium, intracellular Ca^{2+}stores were emptied with CPA (20 μM) and Hist (100 μM) in HeLa cells (panels A, B), or CPA (20 μM) and Cch (100 μM) in HEK293 cells (panels C, D); $CaCl_2$ (1 mM) was then added to measure CCE. Traces are representative of at least 10 independent experiments.

experimental condition (data not shown), excluding, also in this cellular model, any compensatory modification of the total PS level.

Experiments were then designed to look for a correlation between the Ca^{2+} phenotype and the expression level of the mutant protein. Following co-transfection with the photoprotein AEQ, changes in Ca^{2+} responses were analysed in the same cells treated with dox at increasing doses, from 0 to 175 pg/ml (Fig. 5B). In the absence of drug treatment, i.e., in a condition of maximal protein expression, the t-TA HeLa clone overexpressing the PS2-T122R mutant showed a reduction of both Ca^{2+} release from intracellular stores and CCE of about 30% with respect to the control, void vector-transfected cell clone. Increasing the dox concentration up to 150 pg/ml, a condition under which the PS2-T122R protein level was reduced by about 70% (Fig. 5A), Ca^{2+} release from intracellular stores and Ca^{2+} influx became more similar to those found in control cells (15% inhibition compared to controls, Fig. 5B). To further reduce the expression level of PS2-T122R, t-TA HeLa cells were treated with dox at 175 pg/ml, a dose at which only the endogenous PS2 protein was detectable (Fig. 5A). Under this condition, when the mutant protein became undetectable by Western blot analysis, the Ca^{2+} responses were indistinguishable from those of control cells, thus further suggesting a direct correlation between the expression level of the mutant PS2 and Ca^{2+} dysregulation.

As far as PS2-wt is concerned, a similar result was observed, although a less stringent correlation was noticed. In fact, as shown in Fig. 5C, at 150 pg/ml of dox, a dose at which the wt protein was still expressed by t-TA HeLa cells (Fig. 5A), no effect on Ca^{2+} responses was found, thus indicating that higher levels of PS2-wt are required to mimic the action of the mutant form on Ca^{2+} handling.

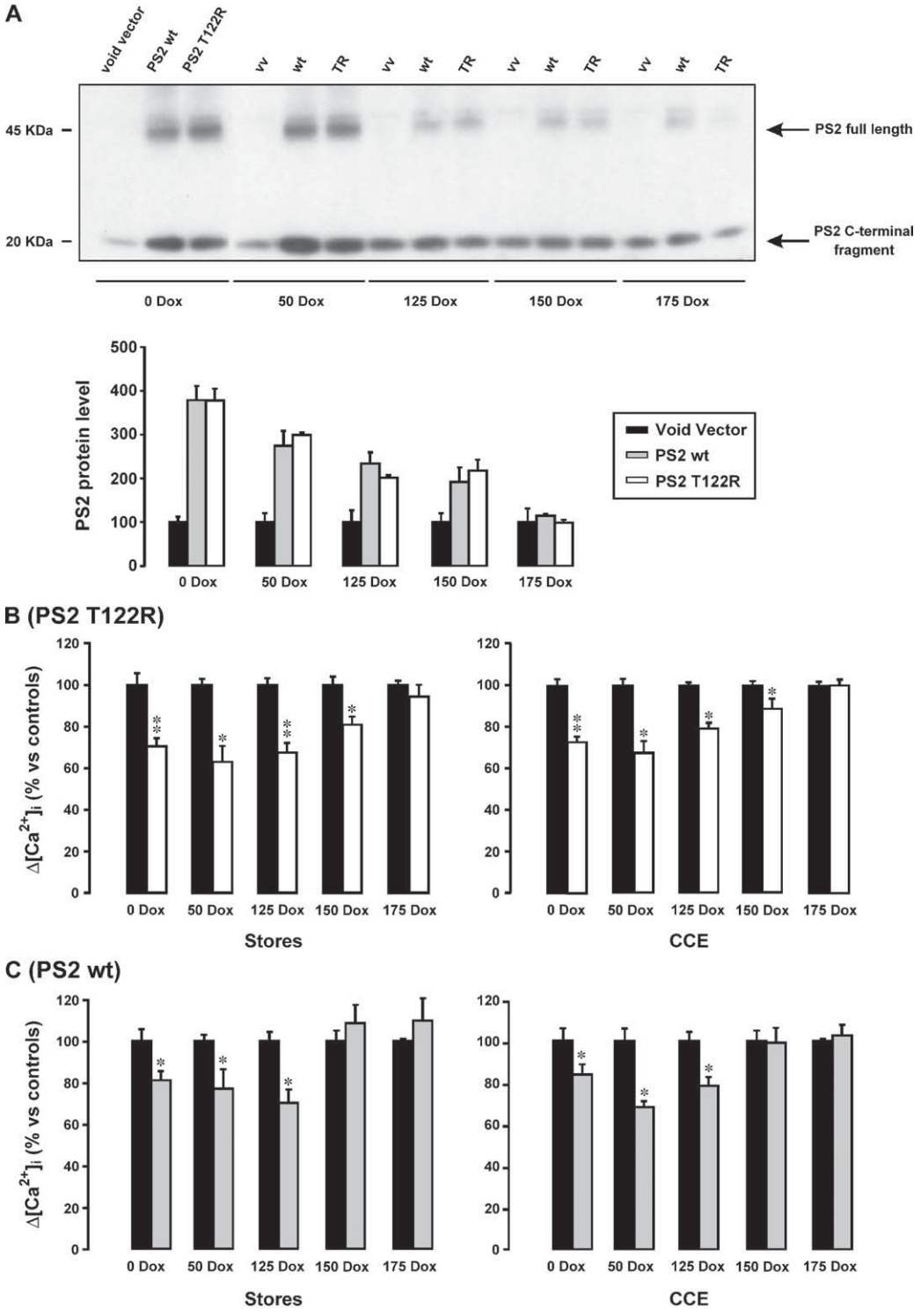
Fig. 5. The entity of Ca^{2+} dysregulation tightly correlates with the PS2 protein expression level. The t-TA HeLa cell line stably expressing the transactivator (see Materials and methods) was transiently transfected with the pMAR void vector, pMAR PS2-wt or -T122R plasmids together with the cDNA for the Ca^{2+} probe AEQ, and analysed for protein expression and Ca^{2+} responses after doxycycline (Dox) treatment. Panel A shows Western blot analysis of void vector (vv)-, PS2-wt (wt)- and PS2-T122R (TR)-transfected t-TA HeLa cells treated with Dox at different doses (0–175 pg/ml). Cell lysates (15 μg per lane) were loaded on 10–20% denaturing Tris–Tricine gel and immunoblotted by using the polyclonal antibody Ab-2 PC235 (Oncogene) that recognises the full-length PS2 protein and its 20-kDa C-terminal proteolitic fragment. For each cell type, densitometric analysis of the bands corresponding to the full length protein is shown in histograms (mean values ± SEM, $n = 3$). Panels B and C show the Ca^{2+} responses recorded with the protocol described in Fig. 4 in corresponding cell batches treated with Dox at the same doses. Panel B shows the $[Ca^{2+}]_i$ increases in PS2-T122R-transfected t-TA HeLa cells due to both intracellular Ca^{2+}release (left) and CCE (right), expressed as a percentage of control values obtained in void vector (vv)-transfected t-TA HeLa (mean ± SEM; n = number of independent experiments, $n = 20$ vv, $n = 15$ PS2-T122R; * = $P < 0.05$, Student's t test). Panel C shows the same values obtained in PS2-wt-transfected t-TA HeLa cells ($n = 8$ vv, $n = 11$ PS2-wt overexpressing cells; ** = $P < 0.001$, * = $P < 0.05$, Student's t test).

Discussion

Impairment of cellular Ca^{2+} signalling has been causally associated to ageing and several neuronal disorders, including AD (Hartmann et al., 1994; Mattson et al., 2000).

The discovery that different PS1 mutations, which include the majority of familial cases of AD, enhance the Ca^{2+} responses caused by activation of $InsP_3$- and ryanodine-sensitive Ca^{2+} channels, led to the hypothesis that altered proteolytic APP processing and increased neuronal vulnerability to excitotoxicity and apoptosis are likely consequences of an "ER Ca^{2+} overload" (Chan et al., 2000; Guo et al., 1996, 1997; Herms et al., 2003; Leissring et al., 2000a,b; Popescu et al., 2004; Schneider et al., 2001; Smith et al., 2002; Stutzmann et al., 2004; for a review, see also LaFerla, 2002).

We previously showed that the FAD-linked PS2 mutation M239I exerts an opposite effect on Ca^{2+} release from intracellular stores (Zatti et al., 2004). This rather unexpected result, obtained in



different experimental models, forced us to investigate other FAD-linked PS2 mutations to check whether it represents an odd on the common theme or, vice versa, when shared by other PS2 mutations, it may contribute to explain differences between the clinical phenotypes associated to PS2 and PS1 mutations.

An Italian pedigree with an autosomal dominant trait of dementia associated with a novel PS2 mutation (T122R) was recently described by our group (Binetti et al., 2003). Among first-degree siblings, we described two monozygotic twins (III-2, III-3) carrying the mutation: interestingly, at the time of first evaluation and fibroblasts collection subject III-2 was affected, while her sister (III-3) presented a normal clinical examination and did not show any cognitive deficits, which, however, appeared only 3 years later.

The aim of the present study was to determine the influence on cellular Ca^{2+} signalling of this new FAD-linked PS2 mutant, testing the possibility that different FAD-associated PS2 mutations share a common feature on cellular Ca^{2+} responses, although opposite to that reported for the large majority of PS1 mutations. In addition, taking advantage of the twins' phenotype, we had the possibility to study the linkage between the defects in Ca^{2+} handling and the presence of clinical symptoms.

We first analysed primary fibroblasts from two monozygotic twin sisters carrying the PS2-T122R mutation and from healthy control subjects related or not to the family. We found that cells from subjects carrying the PS2-T122R mutation showed a reduction in the Ca^{2+} content of intracellular stores compared to control cells (both family related and unrelated controls). We previously demonstrated that an altered ER Ca^{2+} content is a typical feature of the familial form of the disease linked to a PS2 mutation since this modification was not present in fibroblasts of sporadic AD patients (Zatti et al., 2004). The data here presented (obtained with cells carrying a novel PS2 mutation) extend the result previously obtained with PS2-M239I, strongly suggesting the possibility that a reduced Ca^{2+} release is a common feature of FAD-linked PS2 mutations.

As far as CCE is concerned, the PS2-T122R cells showed a Ca^{2+} influx pathway activated by store depletion that was reduced, both in amplitude and activation rate, when compared to CCE of control fibroblasts. In this regard, PS2-T122R differs from PS2-M239I, the previously described PS2 mutant, since fibroblasts from patients carrying the latter mutation did not show significant alterations in CCE compared to control cells (Zatti et al., 2004).

Cellular Ca^{2+} handling was affected by PS2-T122R also in two other different cell models: HeLa and HEK293 cells overexpressing the PS2 mutant. In both cell types, Ca^{2+} release from intracellular stores and CCE were significantly reduced, reinforcing the idea that this mutation influences cellular Ca^{2+} responses.

By comparing the amount of reduction, respectively, in ER Ca^{2+} content and CCE between the two different mutations (M239I vs. T122R), we can also obtain another information. In fact, in primary fibroblasts, the reduction of the ER Ca^{2+} content by PS2-T122R was minor compared to that caused by PS2-M239I (about 25% vs. 38% reduction, see also Zatti et al., 2004), whereas CCE was significantly affected only by PS2-T122R. Therefore, we can hypothesise first that the effect on Ca^{2+} stores cannot be a direct consequence of a reduced CCE and second that the latter can be regarded as an additional PS2-T122R target, whose effect on the clinical phenotype needs to be further investigated. Remarkably, the comparison of these two Italian pedigrees shows a clinical phenotype characterised by a different age of onset ranging from 55 to 60 years for PS2-T122R family and from 45 to 69 years for PS2-M239I family; in addition, the progression of the disease in the PS2-T122R pedigree is more rapid (Binetti et al., 2003; Finckh et al., 2000b; Signorini et al., 2004).

The relevant role that PS2-T122R plays on cellular Ca^{2+} handling is also strengthened by the results obtained with the tetracycline-inducible expression system. By employing this approach in HeLa cells, we provide evidence of a direct correlation between the expression level of the mutant protein and the Ca^{2+} phenotype. The reduction in both Ca^{2+} release from intracellular stores and CCE closely followed the increase in the expression level of PS2-T122R. Noteworthy, by this experimental approach, we could also demonstrate that the PS2-wt mimics the effects elicited by the mutant form on Ca^{2+} handling only when it is largely overexpressed. In fact, at variance with mutant protein, the PS2-wt was ineffective when expressed at a middle level. On the other hand, at lower drug doses, i.e., at high levels of protein expression, the PS2-wt induced an effect on ER Ca^{2+} content and CCE similar to that evoked by PS2-T122R, although less pronounced. These results are in agreement with our previous findings (Zatti et al., 2004) and fit with the idea that the effect on Ca^{2+} homeostasis of the majority of FAD-associated PS mutations are presumably linked to a toxic "gain of function" (Haass and De Strooper, 1999).

It has previously been reported that, in stably transfected cells, expression of exogenous PS1 or PS2 is accompanied by a compensatory decrease in the steady state levels of endogenous PS proteins, consistently with a model in which the abundance of PS1 and PS2 is regulated by competition for limiting cellular factor(s) (Thinakaran et al., 1997). In our study, we showed that all the cell models expressing the PS2-T122R mutation (primary fibroblasts, transiently transfected HeLa and HEK293 cells and the inducible HeLa cell clone) present PS1 levels comparable to those of control cells, thus excluding any influence of this parameter on the here described modifications of cellular Ca^{2+} responses.

Alterations in Ca^{2+} homeostasis have been suggested to be an early manifestation of AD since a large body of 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 (Etcheberrigaray et al., 1998) or extracellular Aβ pathology (Guo et al., 1999; Leissring et al., 2000a). In the present study, we analysed fibroblasts from two monozygotic twins carrying the PS2-T122R mutation: at the time of tissue collection, twin III-2 showed evident clinical signs of disease, while twin III-3 presented a normal clinical examination. Nevertheless, evidence arising from genetic screening, functional neuroimaging, and other biological parameters unravelled, in this latter subject, the beginning of a neurodegenerative process (Binetti et al., 2003). To date, subject III-3 is developing dementia.

We here show evidence that the PS2-T122R mutation causes a reduction in ER Ca^{2+} content and substantial CCE inhibition. Interestingly, we found that Ca^{2+} responses of primary fibroblasts from the two monozygotic twins were similarly affected in spite of the different disease onset, thus suggesting that the FAD-associated Ca^{2+} alteration due to PS2-T122R is an early event in AD pathogenesis and might be a prognostic marker for dementia in asymptomatic carriers.

In conclusion, data from three different cell models and multiple experimental approaches strongly suggest that the PS2-T122R mutation, similarly to PS2-M239I, negatively affects the

ER Ca^{2+} content by favouring a depletion rather than an overload of the organelle, a result opposite to that found with the majority of FAD-linked PS1 mutations. The observation that mutations in PS1 or PS2 affect the Ca^{2+} content of intracellular store in an opposite way is apparently in total contradiction with the hypothesis, until recently mainly based on results obtained with PS1 mutants, that neuronal degeneration in these forms of AD is dependent on Ca^{2+} overload. One may be tempted to conclude that the alterations caused by PS mutations on Ca^{2+} homeostasis are side effects, with no impact on the pathogenesis of neuronal degeneration. We here propose a more conservative working hypothesis that is consistent not only with both results, but also with the more recent concepts concerning the role of Ca^{2+} store in cell death (Orrenius et al., 2003). It is well known that PS mutations cause different alterations in γ-secretase activity, tau phosphorylation, Aβ deposition, etc., and the degree of these modifications is directly correlated with the strength of the primary neurotoxic stimulus; on the other hand, through molecular mechanisms yet unknown, PS1 mutations increase the Ca^{2+} content of intracellular stores, while PS2 mutations have the opposite effect. As demonstrated in several models of cell death, a reduction in the ER Ca^{2+} content is partially protective against a variety of cytotoxic insults, while an increase in Ca^{2+} within the stores exacerbates the efficacy of the death stimuli (Pinton et al., 2001; Scorrano et al., 2003; Suen et al., 2003). In other words, we propose that in FAD Ca^{2+} acts as a cofactor rather than as the causative agent. According to this hypothesis, mutations in PS that reduce the ER Ca^{2+} content, would be partially protective, while mutations that increase Ca^{2+} in the stores aggravate the primary insult. It is interesting to note not only that in general the clinical phenotype of FAD patients affected by PS1 mutations is more aggressive and characterised by an earlier age of onset compared to that of patients affected by PS2 mutations (Finckh et al., 2000b; Mann et al., 1997; Sherrington et al., 1996), but also that, of the two PS2 mutations we have analysed, fibroblasts from patients carrying the PS2-M239I show a major reduction of the ER Ca^{2+} content (Zatti et al., 2004) and, correspondingly, the onset and the progression of the disease is relatively late compared to those due to the PS2-T122R mutation (Binetti et al., 2003; Finckh et al., 2000b; Signorini et al., 2004). According to this conservative hypothesis, the onset and the gravity of the symptoms in FAD would depend on a delicate balance between the intensity of the primary insult and the secondary effect on Ca^{2+} handling. It is clear that this is still a working hypothesis that could be experimentally tested in the different models.

In summary, the finding that two PS2 mutations, M239I and T122R, reduce instead of increase the Ca^{2+} content of intracellular stores suggests that Ca^{2+} defects in AD play a modulatory rather than a causative role in the pathogenesis of the disease; the extent of alteration in Ca^{2+} handling may profoundly affect prominent clinical features such as onset, penetrance and progression.

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References

Binetti, G., Signorini, S., Squitti, R., Alberici, A., Benussi, L., Cassetta, E., Frisoni, G.B., Barbiero, L., Feudatari, E., Nicosia, F., Testa, C., Zanetti, O., Gennarelli, M., Perani, D., Anchisi, D., Ghidoni, R., Rossini, P.M., 2003. Atypical dementia associated to a novel presenilin-2 mutation. Ann. Neurol. 54, 832–836.

Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., Rizzuto, R., 1995. Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$): a critical evaluation. J. Biol. Chem. 270, 9896–9903.

Chan, S.L., Mayne, M., Holden, C.P., Geiger, J.D., Mattson, M.P., 2000. Presenilin-1 mutations increase levels of ryanodine receptors and calcium release in PC12 cells and cortical neurons. J. Biol. Chem. 275, 18195–18200.

Cruts, M., van Duijin, C.M., Backhovens, H., Van den Broeck, M., Wehnert, A., Serneels, S., Sherrington, R., Hutton, M., Hardy, J., St George-Hyslop, P.H., Hofman, A., Van Broeckhoven, C., 1998. Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease. Hum. Mol. Genet. 7, 43–51.

Etcheberrigaray, R., Hirashima, N., Nee, L., Prince, J., Govoni, S., Racchi, M., Tanzi, R.E., Alkon, D.L., 1998. Calcium responses in fibroblasts from asymptomatic members of Alzheimer's disease families. Neurobiol. Dis. 5, 37–45.

Ezquerra, M., Lleo, A., Castellvi, M., Queralt, R., Santacruz, P., Pastor, P., Molinuevo, J.L., Blesa, R., Oliva, R., 2003. A novel mutation in the PSEN2 gene (T430M) associated with variable expression in a family with early-onset Alzheimer disease. Arch. Neurol. 60, 1149–1151.

Finckh, U., Muller-Thomsen, T., Mann, U., Eggers, C., Marksteiner, J., Meins, W., Binetti, G., Alberici, A., Hock, C., Nitsch, R.M., Gal, A., 2000a. High prevalence of pathogenic mutations in patients with early-onset dementia detected by sequence analyses of four different genes. Am. J. Hum. Genet. 66, 110–117.

Finckh, U., Alberici, A., Antoniazzi, M., Benussi, L., Fedi, V., Giannini, C., Gal, A., Nitsch, R.M., Binetti, G., 2000b. Variable expression of Familial Alzheimer's disease associated with an M239I mutation in the presenilin 2 gene. Neurology 54, 2006–2008.

Folstein, M.F., Folstein, S.E., McHugh, P.R., 1975. Mini-mental state: a practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. 12, 189–198.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., et al., 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704–706.

Govoni, S., Bergamaschi, S., Racchi, M., Battaini, F., Binetti, G., Bianchetti, A., Trabucchi, M., 1993. Cytosol protein kinase C down-regulation in fibroblasts from Alzheimer's disease patients. Neurology 43, 2581–2586.

Guo, Q., Furukawa, K., Sopher, B.L., Pham, D.G., Xie, J., Robinson, N., Martin, G.M., Mattson, M.P., 1996. Alzheimer's PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid β-peptide. Neorochemistry 8, 379–383.

Guo, Q., Sopher, B.L., Furukawa, K., Pham, D.G., Robinson, N., Martin, G.M., Mattson, M.P., 1997. Alzheimer's presenilin mutation sensitizes

neural cells to apoptosis induced by trophic factor withdrawal and amyloid beta-peptide: involvement of calcium and oxyradicals. J. Neurosci. 17, 4212–4222.

Guo, Q., Fu, W., Sopher, B.L., Miller, M.W., Ware, C.B., Martin, G.M., Mattson, M.P., 1999. Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. Nat. Med. 5, 101–106.

Haass, C., De Strooper, B., 1999. The presenilins in Alzheimer's disease—Proteolysis holds the key. Science 286, 916–919.

Hartmann, H., Eckert, A., Muller, W.E., 1994. Disturbances of the neuronal calcium homeostasis in the aging nervous system. Life Sci. 55, 2011–2018.

Herms, J., Schneider, I., Dewachter, I., Caluwaerts, N., Kretzschmar, H., Van Leuven, F., 2003. Capacitive calcium entry is directly attenuated by mutant presenilin-1, independent of the expression of the amyloid precursor protein. J. Biol. Chem. 278, 2484–2489.

Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., Mucke, L., 1999. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse model. Proc. Natl. Acad. Sci. U. S. A. 96, 3228–3233.

Hsiao, K., Chapman, P., Nilsen, S., Ekman, C., Harigaya, Y., Younkin, S., Yang, F., Cole, G., 1996. Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science 274, 99–102.

LaFerla, F.M., 2002. Calcium dyshomeostasis and intracellular signaling in Alzheimer's disease. Nat. Rev., Neurosci. 3, 862–872.

Lai, M.T., Chen, E., Crouthamel, M.C., DiMuzio-Mower, J., Xu, M., Huang, Q., Price, E., Register, R.B., Shi, X.P., Donoviel, D.B., Bernstein, A., Hazuda, D., Gardell, S.J., Li, Y.M., 2003. Presenilin-1 and presenilin-2 exhibit distinct yet overlapping gamma-secretase activities. J. Biol. Chem. 278, 22475–22481.

Leissring, M.A., Akbari, Y., Fanger, C.M., Cahalan, M.D., Mattson, M.P., LaFerla, F.M., 2000a. Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. J. Cell Biol. 149, 793–797.

Leissring, M.A., Yamasaki, T.R., Wasco, W., Buxbaum, J.D., Parker, I., LaFerla, F.M., 2000b. Calsenilin reverses presenilin-mediated enhancement of calcium signaling. Proc. Natl. Acad. Sci. U. S. A. 97, 8590–8593.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K., et al., 1995. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 269, 973–977.

Mann, D.M., Iwatsubo, T., Nochlin, D., Sumi, S.M., Levy-Lahad, E., Bird, T.D., 1997. Amyloid (Abeta) deposition in chromosome 1-linked Alzheimer's disease: the Volga German families. Ann. Neurol. 41, 52–57.

Mattson, M.P., LaFerla, F.M., Chan, S.L., Leissring, M.A., Shepel, P.N., Geiger, J.D., 2000. Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. Trends Neurosci. 23, 222–229.

Orrenius, S., Zhivotovsky, B., Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. Nat. Rev., Mol. Cell Biol. 4, 552–565.

Pinton, P., Ferrari, D., Rapizzi, E., Di Virgilio, F., Pozzan, T., Rizzuto, R., 2001. The Ca^{2+} concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. EMBO J. 20, 2690–2701.

Popescu, B.O., Cedazo-Minguez, A., Benedikz, E., Nishimura, T., Winblad, B., Ankarcrona, M., Cowburn, R.F., 2004. Gamma-secretase activity of presenilin 1 regulates acetylcholine muscarinic receptor-mediated signal transduction. J. Biol. Chem. 279, 6455–6464.

Putney Jr., J.W., 2000. Presenilins, Alzheimer's disease, and capacitative calcium entry. Neuron 27, 411–412.

Rogaev, E.I., Sherrington, R., Rogeava, E.A., Levesque, G., Ikeda, M., Liang, Y., et al., 1995. Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376, 775–778.

Schneider, I., Reversé, D., Dewachter, I., Ris, L., Caluwaerts, N., Kuipéri, C., Gilis, M., Geerts, H., Kretzschmar, H., Godaux, E., Moechars, D., Van Leuven, F., Herms, J., 2001. Mutant presenilins disturb neuronal calcium homeostasis in the brain of transgenic mice, decreasing the threshold for excitotoxicity and facilitating long-term potentiation. J. Biol. Chem. 276, 11539–11544.

Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T., Korsmeyer, S.J., 2003. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300, 135–139.

Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levasque, G., Ikeda, M., et al., 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375, 754–760.

Sherrington, R., Froelich, S., Sorbi, S., Campion, D., Chi, H., Rogaeva, E.A., Levesque, G., Rogaev, E.I., Lin, C., Liang, Y., Ikeda, M., Mar, L., Brice, A., Agid, Y., Percy, M.E., Clerget-Darpoux, F., Piacentini, S., Marcon, G., Nacmias, B., Amaducci, L., Frebourg, T., Lannfelt, L., Rommens, J.M., St George-Hyslop, P.H., 1996. Alzheimer's disease associated with mutations in presenilin 2 is rare and variably penetrant. Hum. Mol. Genet. 5, 985–988.

Signorini, S., Ghidoni, R., Barbiero, L., Benussi, L., Binetti, G., 2004. Prevalence of pathogenic mutations in an Italian clinical series of patients with familial dementia. Curr. Alzheimer Res. 1, 215–218.

Smith, I.F., Boyle, J.P., Vaughan, P.F.T., Pearson, H.A., Cowburn, R.F., Peers, C.S., 2002. Ca^{2+} stores and capacitative Ca^{2+} entry in human neuroblastoma (SH-SY5Y) cells expressing a familial Alzheimer's disease presenilin-1 mutation. Brain Res. 949, 105–111.

Stutzmann, G.E., Caccamo, A., LaFerla, F.M., Parker, I., 2004. Dysregulated IP_3 signaling in cortical neurons of knock-in mice expressing an Alzheimer's-linked mutation in presenilin 1 results in exaggerated Ca^{2+} signals and altered membrane excitability. J. Neurosci. 24, 508–513.

Suen, K.-C., Lin, K.-F., Elyaman, W., So, K.-F., Chuen-Chung Chang, R., Hugon, J., 2003. Reduction of calcium release from endoplasmic reticulum could only provide partial neuroprotection against beta-amyloid peptide toxicity. J. Neurochem. 87, 1413–1426.

Takeda, K., Araki, W., Tabira, T., 2004. Enhanced generation of intracellular Abeta42 amyloid peptide by mutation of presenilins PS1 and PS2. Eur. J. Neurosci. 19, 258–264.

Tedde, A., Nacmias, B., Ciantelli, M., Forleo, P., Cellini, E., Bagnoli, S., Piccini, C., Caffarra, P., Ghidoni, E., Paganini, M., Bracco, L., Sorbi, S., 2003. Identification of new presenilin gene mutations in early-onset familial Alzheimer disease. Arch. Neurol. 60, 1541–1544.

Thinakaran, G., Harris, C.L., Ratovitski, T., Davenport, F., Slunt, H.H., Price, D.L., Borchelt, D.R., Sisodia, S.S., 1997. Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. J. Biol. Chem. 272, 28415–28422.

Wells, K.D., Foster, J.A., Moore, K., Pursel, V.G., Wall, R.J., 1999. Codon optimization, genetic insulation, and an rtTA reporter improve performance of the tetracycline switch. Transgenic Res. 8, 371–381.

Zatti, G., Ghidoni, R., Barbiero, L., Binetti, G., Pozzan, T., Fasolato, C., Pizzo, P., 2004. The presenilin 2 M239I mutation associated with Familial Alzheimer's Disease reduces Ca^{2+} release from intracellular stores. Neurobiol. Dis. 15/2, 269–278.