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Ca^{2+} dysregulation in neurons from transgenic mice expressing mutant presenilin 2

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Summary

Mutations in amyloid precursor protein (APP), and presenilin-1 and presenilin-2 (PS1 and PS2) have causally been implicated in Familial Alzheimer's Disease (FAD), but the mechanistic link between the mutations and the early onset of neurodegeneration is still debated. Although no consensus has yet been reached, most data suggest that both FAD-linked PS mutants and endogenous PSs are involved in cellular Ca^{2+} homeostasis. We here investigated subcellular Ca^{2+} handling in primary neuronal cultures and acute brain slices from wild type and transgenic mice carrying the FAD-linked PS2-N141I mutation, either alone or in the presence of the APP Swedish mutation. Compared with wild type, both types of transgenic neurons show a similar reduction in endoplasmic reticulum (ER) Ca^{2+} content and decreased response to metabotropic agonists, albeit increased Ca^{2+} release induced by caffeine. In both transgenic neurons, we also observed a higher ER–mitochondria juxtaposition that favors increased mitochondrial Ca^{2+} uptake upon ER Ca^{2+} release. A model is described that integrates into a unifying hypothesis the contradictory effects on Ca^{2+} homeostasis of different PS mutations and points to the relevance of these findings in neurodegeneration and aging.

Key words: Alzheimer; calcium; endoplasmic reticulum; mitochondria; neurons; presenilin.

Introduction

A critical role of cellular Ca^{2+} in neuronal aging was initially proposed in the late 1980s, in the form of the 'Ca^{2+} hypothesis' of neuronal aging (Khachaturian, 1987). Accordingly, brain aging is associated to an increasing dysregulation of Ca^{2+} homeostasis, which results in a sustained elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]$), excitotoxicity and neuronal loss. It is now clear that during the initial phases of aging, intracellular Ca^{2+} dynamics undergo numerous and more subtle changes that culminate in synaptic dysfunctions and memory impairment, even in the absence of overt neuronal loss (Toescu & Verkhratsky, 2007). Such subtle alterations are dramatically increased and anticipated in age of onset in pathological forms of neurodegeneration and, in particular, in Alzheimer's Disease (AD) (Khachaturian, 1994; Toescu & Vreugdenhil, 2010; Camandola & Mattson, 2011). Noteworthy, not only Ca^{2+} dysregulation is a common feature of the aged and demented brain, but elevated amyloid deposition, a histological characteristic of AD, can be also found in normal aged people. The mechanisms that determine the transition from normal aging to mild cognitive impairment and eventually to overt dementia are still matter of intense investigation. Understanding the precocious phases of Ca^{2+} mishandling in AD mouse models might shed light on the molecular mechanisms involved in this transition.

The large majority of AD mouse models are transgenic (tg) mice based on one or two of the three genes that carry the autosomal dominant mutations found in the familial form of the disease (FAD): that is, *APP, PSEN1,* and *PSEN2*, coding for APP, PS1, and PS2, respectively. FAD represents less than 5% of AD cases, but the causal link between the disease and the gene mutations is firmly established. Conversely, all other AD cases are sporadic, with APOE-ε4 and age as major risk factors, but the pathogenic mechanisms have not been clarified yet.

In mammals, PSs are ubiquitously expressed and constitute the catalytic core of γ-secretases, endoproteases which, in concert with β-secretases, produce amyloid-β (Aβ) peptides from APP (Sisodia & St George-Hyslop, 2002). According to the amyloid hypothesis, mutations in APP and PSs alter the relative proportion of Aβ peptides and promote the generation of the more aggregation-prone Aβ42, thus favouring the early onset of FAD by amyloid toxicity (Selkoe, 1998). More recently, FAD-linked mutations (in particular those linked to PS1) have strictly been correlated with altered Ca^{2+} signaling (La Ferla, 2002). The key question is whether alterations in Ca^{2+} homeostasis are the primary cause of neuronal dysfunction, are secondary to other molecular defects (especially Aβ42 production) or are simply concomitant events that exacerbate the disease. In cultured model cells, a large body of evidence suggests that PSs regulate Ca^{2+} homeostasis independently of γ-secretase activity and toxic Aβ peptide generation. Specifically, it has been suggested that FAD-linked PS mutations increase endoplasmic reticulum (ER) Ca^{2+} accumulation, resulting in exaggerated ER Ca^{2+} release (Guo *et al.*, 1996). The so-called Ca^{2+} overload hypothesis was reinforced in the latest years by data suggesting that PSs, as holoproteins, may work as endogenous ER Ca^{2+} leak channels, whereas FAD-linked PS mutants, by reducing the channel conductance, favor the ER Ca^{2+} overload (La Ferla, 2002; Thinakaran & Sisodia, 2006; Bezprozvanny & Mattson, 2008). Along the same line, neurons from tg mice expressing FAD-linked PS1 mutations over-express the ryanodine receptor (Ry-R), an intracellular Ca^{2+} release channel, thus increasing the likelihood of exaggerated Ca^{2+} release and neuronal damage (Stutzmann *et al.*, 2007; Camandola & Mattson, 2011). These conclusions, however, have been challenged by a number of recent studies, in particular: (i) direct measurements of IP_3-R opening probability reveal that expression of FAD-PS mutants increases the channel sensitivity to basal IP_3 levels (Cheung *et al.*, 2008); (ii) the use of genetically-

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encoded ER Ca^{2+} sensors demonstrates that different model cells, stably or transiently expressing FAD-linked PS1 and PS2 mutants, have a normal or reduced ER Ca^{2+} level (Zatti *et al.*, 2006; McCombs *et al.*, 2010). In the case of PS2 mutants, this latter reduction was because of inhibition of SERCA activity and increase in Ca^{2+} leak (Brunello *et al.*, 2009); (iii) finally, in a thorough investigation of wild type (wt) and FAD-mutant PS1 expressing cells, it was recently demonstrated that neither wt nor PS1 mutants affect ER Ca^{2+} uptake, leak and steady state level (Shilling *et al.*, 2012).

In this study, we focus our attention on Ca^{2+} dysregulation in tg mouse models based on a FAD-mutant PS2, *that is*, the single tg mouse line PS2.30H, homozygous for PS2-N141I, and the double tg mouse line B6.152H, homozygous for both PS2-N141I and APP Swedish (APPswe) K670N, M671L mutant (Ozmen *et al.*, 2009). By employing different Ca^{2+} imaging techniques on primary neuronal cultures and acute brain slices, we demonstrate that Ca^{2+} homeostasis is similarly altered in both single and double tg mice; such alterations are qualitatively and quantitatively similar to those reported in human FAD fibroblasts and cell lines over-expressing PS2 mutants (Zatti *et al.*, 2004, 2006). In both tg mice, neurons have similar reductions in total Ca^{2+} store content and response to IP_3-generating agonists, albeit different Aβ levels in their brains. Furthermore, both tg neurons show increased response to caffeine and increased Ca^{2+} excitability. By employing recombinant fluorescent probes in neuronal cultures from tg mice, we also demonstrate an increased mitochondrial capability to take up Ca^{2+}, owing to a greater ER–mitochondria juxtaposition. In these tg mice, dysregulation of neuronal Ca^{2+} stores occurs at an early age and appears to depend directly on the mutant PS2 itself and not on PS2-dependent APP processing or total Aβ levels. A pathogenic model that accounts for the different findings with PS1 and PS2 mutations and centerd on mitochondria Ca^{2+} toxicity is proposed.

Results

PS2 and APP expression levels

In this work, we have taken advantage of single (PS2.30H) and double (B6.152H) tg mouse lines expressing PS2-N141I, respectively, in the absence or presence of APPswe; for clarity, these lines were here named PS2-N141I and PS2APP, respectively. The PS2 mutant is expressed ubiquitously, whereas the APP mutant is expressed in neurons and thymocytes (Ozmen *et al.*, 2009). Figure 1 shows that, in brain homogenates from 2-week-old tg mice and in primary neuronal cultures at 10–12 DIV, the amount of total PS2, compared with that of wt mice, was 1.8- to 2.2-fold larger in both genotypes. Accordingly, to a first approximation, the ratio wt⁄mutated PS2 is approximately 1, *that is*, similar to that found in FAD heterozygous patients. Regarding APP, in mouse brains and primary neuronal cultures at 10-12 DIV from double tg mice, its level was at least twice that of controls (2.3 ± 0.6 a.u., $P < 0.03$, $n = 9$ animals, and 2.90 ± 0.14 a.u., $P < 0.01$, $n = 3$ cultures, mean ± SEM), while in brains and cultures from single tg mice, it was indistinguishable from that of wt animals (not shown).

Ca^{2+} release induced by IP_3-generating agonists

We have shown previously that, in rat primary neurons loaded with Fura-2, cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) rises, because of intracellular release, are substantially increased by a short exposure to KCl (Zatti *et al.*, 2006). Similarly, when cultured mouse neurons were first challenged with KCl (30 mM) and then, after extracellular Ca^{2+} and KCl removal, with the

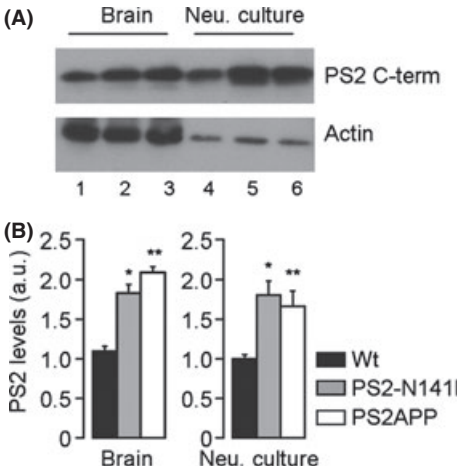

Fig. 1 Presenilin-2 (PS2) levels in whole brains and neuronal cultures from tg mice. (A) 20 μg of brain homogenates (lanes 1,2,3) and neuronal (neu) cultures (lanes 4,5,6) from wt (1,4), PS2-N141I (2,5) or PS2APP (3,6) mice were blotted and probed for PS2 expression. (B) Statistics of PS2 expression levels in whole brain homogenates and neuronal cultures. Values were first normalized by their internal actin levels and then to those of wt samples. Data are mean ± SEM of nine independent brain samples for each genotype, or three independent cultures run in triplicate.

muscarinic agent charbacol (CCH, 0.5 mM), a net increase in $[Ca^{2+}]_c$ was observed in the majority of neurons; on the contrary, only a minority of cells responded with a Ca^{2+} rise when the agonist was added before KCl (Fig. S1A). The KCl prepulse protocol was therefore adopted to investigate the amplitude of Ca^{2+} mobilization from the stores in wt and tg neurons. In most neurons (of each genotype), the resting $[Ca^{2+}]_c$ was stable with rare spontaneous Ca^{2+} spikes, which were completely abolished by removal of Ca^{2+} from the medium (not shown). Figure 2A

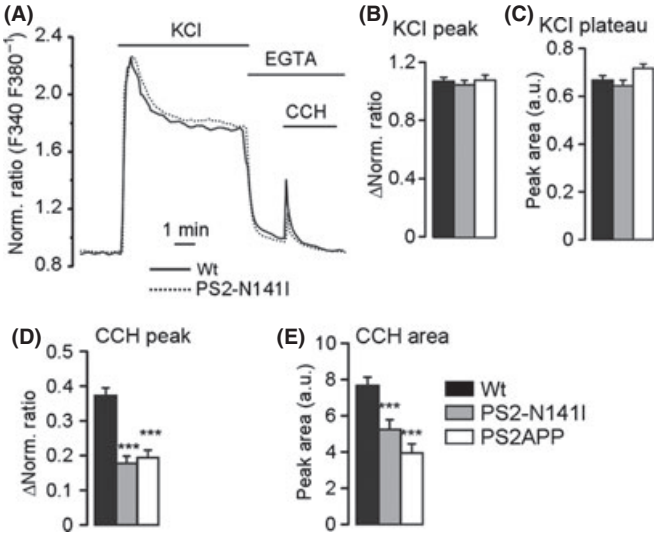

Fig. 2 Reduced Ca^{2+} release in response to IP_3-generating agonists in tg mice. (A) Representative traces of wt and presenilin-2 (PS2)-N141I neurons (10-12DIV) bathed in mKRB, exposed first to KCl (30 mM) in the same medium and then to CCH (0.5 mM) in a Ca^{2+}-free, EGTA (0.5 mM)-containing mKRB. (B,C) Bars represent the average KCl peak and plateau values, above the baseline (mean ± SEM, $n = 86$ wt; $n = 45$ PS2-N141I; $n = 34$ PS2APP). (D,E) Average peak and area values, above the baseline, in response to CCH (0.5 mM) (mean ± SEM, $n = 36$ wt; $n = 20$ PS2-N141I; $n = 21$ PS2APP). Only neurons responding to CCH were included in this calculation.

shows that addition of KCl (30 mM) caused a rapid increase in $[Ca^{2+}]_c$ that remained elevated until perfusion with a Ca^{2+} -free mKRB containing EGTA (0.5 mM). Figure 2B,C shows that no significant difference was observed between neurons from wt, PS2-N141I or PS2APP mice in terms of KCl-induced peaks and plateaus. In contrast, substantial differences were observed between tg and wt mice upon CCH addition. In wt neurons, the average peak and area for $[Ca^{2+}]_c$ rises in response to CCH were 0.37 ± 0.02 $\Delta R/R_0$ and 7.68 ± 0.45 arbitrary units (a.u) (mean ± SEM, n = 36), respectively. These values were significantly reduced in neurons from both tg mice: 52% and 31% decrease in peak and area, respectively, in single tg, and 44% and 49% decrease in the same parameters in double tg neurons (Fig. 2D,E). When the metabotropic glutamate receptor agonist, (*S*)-3,5-dihydroxyphenylglycine (DHPG, 10 μM) was used as a stimulus, peak and area values were similar to those evoked by CCH in each genotype. Accordingly, in tg neurons also the response to DHPG was reduced in peak and area values: 34% and 37% in single tg and 50% and 41% in double tg neurons, respectively.

Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores

Ca^{2+} release from stores in neurons depends not only on IP_3-Rs, but also on Ry-Rs. Figure 3 shows that, in wt neurons, the average peak and area in response to caffeine (20 mM) were 0.14 ± 0.01 $\Delta R/R_0$ and 1.90 ± 0.23 a.u. (mean ± SEM, n = 15), respectively. Despite the reduced response to IP_3 producing agonists, neurons from both tg mice exhibited a significant increase in Ca^{2+} release upon caffeine stimulation, *that is,* the average percentage increases in peak and area were 38% and 96%, respectively, in single tg, and 38% and 76%, respectively, in double tg neurons (Fig. 3A–C). It is worth noting that, on average, in wt neurons the peak $[Ca^{2+}]_c$ elicited by caffeine was about half that caused by CCH or DHPG and, as expected, it was completely inhibited by 1 h pre-incubation with ryanodine (20 μM) (not shown).

Total Ca^{2+} content of intracellular stores

The reduced response to IP_3-generating agonists in tg mouse neurons could be due to reduced IP_3 generation, reduced IP_3-R density/sensitivity or reduced Ca^{2+} content in the stores (or a combination of these mechanisms). To evaluate the total Ca^{2+} content of intracellular stores in the cultured neurons, we took advantage of the Ca^{2+} ionophore ionomycin that releases Ca^{2+} from all, non acidic, intracellular stores, independently of IP_3 generation. When ionomycin (1 μM) was added in a Ca^{2+}-free, EGTA-containing medium after KCl treatment, the average peak and area of wt cortical neurons were 0.65 ± 0.03 $\Delta R/R_0$ and 23.0 ± 1.35 a.u. (mean ± SEM, n = 16), respectively (see Fig. 3D). In neurons from single and double tg mice, the Ca^{2+} response to ionomycin was significantly reduced (about 35% and 28% for peak amplitudes, and 28% and 22% for areas, respectively; Fig. 3E,F). Compared with wt, neurons from PS2-N141I tg mice also lose faster the accumulated Ca^{2+}, as revealed by applying ionomycin in Ca^{2+} -free, EGTA-containing mKRB at different times after KCl treatment (Fig. S1B). The reduction in store Ca^{2+} concentration was finally confirmed by directly monitoring the ER Ca^{2+} level with a D4ER cameleon: after the KCl treatment, the D4ER initial fluorescence ratio value (YFP/CFP), reflecting the steady-state ER Ca^{2+} concentration ($[Ca^{2+}]_{ER}$), was significantly reduced in neurons from single tg mice compared with controls (Fig. 3G). Addition of EGTA resulted in a slow decrease in $[Ca^{2+}]_{ER}$ that was strongly accelerated by addition of the ER Ca^{2+} ATPase blocker cyclopiazonic acid (CPA, 20 μM); further addition of ionomycin (1 μM) caused only a small drop (Fig. 3G), indicating that CPA was sufficient to completely empty the ER Ca^{2+} content, as observed in other cell types (Shilling *et al.*, 2012). The drop caused by EGTA plus CPA was significantly reduced in tg neurons, a result that is perfectly consistent with the Fura-2 data (Figs 3H and S1B).

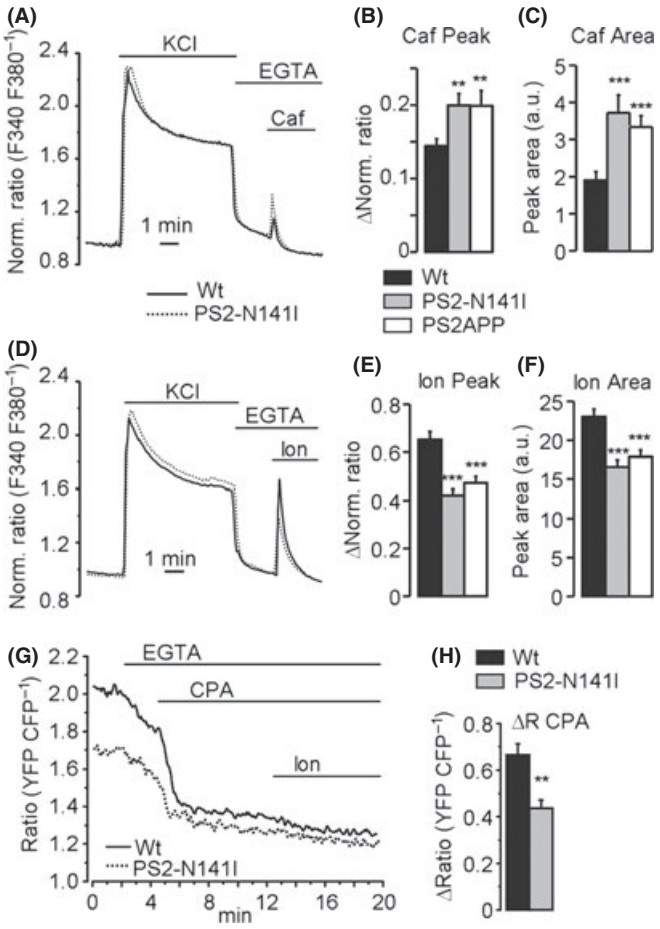

Fig. 3 Ca^{2+} release induced by caffeine is increased in neurons from tg mice despite the reduction in the total store Ca^{2+} content. Representative traces of wt and presenilin-2 (PS2)-N141I neurons (10-12 DIV) bathed in mKRB, exposed first to KCl (30 mM) in the same medium and then to caffeine (20 mM) (A) or ionomycin (1 μM) (D) in a Ca^{2+}-free, EGTA (0.5 mM)-containing mKRB. Average peak and area values, above the baseline in response respectively to caffeine (B,C) or ionomycin (E,F). Values are expressed as mean ± SEM (n = 15 wt; n = 9 PS2-N141I; n = 12 PS2APP for caffeine and n = 16 wt; n = 14 PS2-N141I; n = 12 PS2APP for ionomycin, respectively). (G) Representative traces of wt and PS2-N141I neurons expressing D4ER. Changes in YFP/CFP ratio values were monitored in neurons during the last 2 min of the standard KCl (30 mM) treatment. Upon a 2-min perfusion in Ca^{2+}-free, EGTA containing mKRB, full ER depletion was obtained by addition of CPA (20 μM), followed by ionomycin (1 μM) (H) Statistics of ER Ca^{2+} depletion induced by EGTA and CPA treatment (mean ± SEM, n = 24 wt; n = 20 PS2-N141I).

Ca^{2+} stores in wt and tg mouse neurons *in situ*

Cultured neurons do not reflect the complexity of the brain architecture where interactions among neurons and glial cells occur in their proper environment. Wt and tg neurons were thus compared *in situ*, using acute hippocampal (CA1) slices, loaded with the fluorescent Ca^{2+} indicator Oregon Green 488 BAPTA-1. Neurons and glial cells were distinguished by both their morphology and functionality, as previously described (Fellin

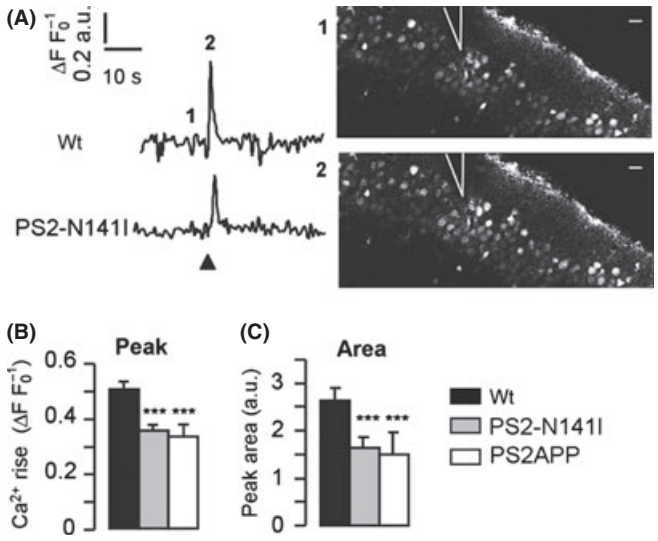

Fig. 4 DHPG-induced Ca^{2+} release is reduced in tg hippocampal slices. (A) Right, representative traces of cells responding to a puff (arrow) of DHPG (100 μM) for wt and presenilin-2 (PS2)-N141I mice. Left, representative CA1 regions in a wt mice before (1, upper panel) and during (2, lower panel) the puff. Pipette position is marked by the void arrow. Scale bar, 25 μm. (B,C) Average $[Ca^{2+}]_c$ peak and area values, measured above the baseline and expressed as $\Delta F/F_0$ in response to DHPG (mean ± SEM, n = 8–11 for each genotype).

et al., 2004). As noticed in cultured neurons, rare spontaneous Ca^{2+} spikes were observed also in neurons *in situ*. Puff application of DHPG (100 μM), in Ca^{2+} -containing ACSF, resulted in a clear and rapid increase in $[Ca^{2+}]_c$ in both wt and tg neurons (Fig. 4A). However, peak and area of the Ca^{2+} responses were significantly reduced in neurons of both tg mice (Fig. 4B,C). On average, the estimated reduction was statistically indistinguishable in the two tg lines, about 30% and 50% for peak and area, respectively. In brain slices, metabotropic agonists, such as DHPG, could affect neuronal Ca^{2+} either directly, by causing IP_3 formation, or indirectly, by inducing Ca^{2+} mobilization in astrocytes followed by glutamate release and finally activation of neuronal ionotropic receptors (Fellin *et al.*, 2004). To check whether the Ca^{2+} rise in neurons was directly dependent on activation of metabotropic receptors expressed in their membranes, the slices were pre-incubated for 5 min with the ionotropic receptor inhibitors 2-amino-5-phosphonopentanoic acid (D-AP5, 25 μM) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo (*f*) quinoxaline-7-sulfonamide (NBQX, 5 μM), together with tetrodotoxin (TTX, 1 μM) to prevent action potential generation. Although this treatment slightly decreased the peak amplitude and area of neuronal Ca^{2+} rises induced by DHPG, the difference between wt and tg neurons was unaffected (see Table 1). On the other hand, slice perfusion with ACSF containing $NiCl_2$ (100 μM), to inhibit Ca^{2+} -permeable channels, had no effect on a subsequent DHPG application (not shown). Furthermore, when the DHPG-induced Ca^{2+} response was tested in Ca^{2+} -free, EGTA-containing medium, the obtained results were consistent with those reported above (see Table 1). Similar findings were obtained when the amplitude of DHPG-induced Ca^{2+} peaks was monitored in astrocytes, *that is*, the average peak amplitude of the Ca^{2+} rises induced by DHPG was larger in wt than in both tg mice (Fig. S2 and Table 1).

No difference in the expression level of IP_3-Rs or SERCA-2 was observed in the brains of the three genotypes (not shown), supporting the conclusion that in neurons and astrocytes of tg mice the reduced response to metabotropic receptor stimulation is primarily due to a reduction in the store Ca^{2+} content [see previous sections and (Zatti *et al.*, 2006; Brunello *et al.*, 2009)].

The data in cultured neurons indicate that, while in cells from tg mice activation of IP_3-Rs results in an attenuated Ca^{2+} peak, the response to Ry-Rs is increased. It has been demonstrated that in other AD mouse models based on PS mutants, the expression level of Ry-Rs is enhanced compared with wt animals (Lee *et al.*, 2006; Stutzmann *et al.*, 2007). Ry-R2 expression was thus verified by Western blot and found to be up-regulated in the brains of both tg mice, compared with controls (Fig. S3); a similar result was observed in cultured neurons (not shown).

Based on these results, we thus tested the effect of caffeine in hippocampal slices. Perfusion with caffeine (20 mM) caused no rapid elevation of cytosolic Ca^{2+} but, after a lag of 5-8 min, it caused a dramatic increase in spontaneous Ca^{2+} spikes. When caffeine was added in the presence of TTX, no rapid increase in $[Ca^{2+}]_c$ nor augmentation in spike activity were observed, suggesting that the latter event is likely due to an effect of caffeine on adenosine receptors (Ribeiro & Sebastiao, 2010). However, a DHPG puff, elicited after 10 min of treatment with caffeine and TTX, resulted in no increase in $[Ca^{2+}]_c$. The total store Ca^{2+} content of neurons in slices could not be assessed by the use of ionomycin, as acute application of the ionophore caused a hardly detectable $[Ca^{2+}]_c$ increase in both neurons and astrocytes, probably because of the inefficient penetration of this highly hydrophobic drug into slices.

Mitochondria implication

Both PSs have been found in mitochondria-associated membranes, domains of tight interaction between mitochondria and ER (Area-Gomez

Table 1 Summary of DHPG-induced Ca^{2+} response in acute slices from wt or tg mice

	Δpeak (F/Fo)			Area Δpeak (F/Fo)		
	ACSF	ACSF + inh	ACSF no Ca^{2+}	ACSF	ACSF + inh	ACSF no Ca^{2+}
Neurons						
wt	0.5 ± 0.03	0.36 ± 0.036	0.46 ± 0.05	2.63 ± 0.26	1.21 ± 0.46	2.32 ± 0.55
PS2-N141I	0.35 ± 0.02***	0.27 ± 0.04*	0.26 ± 0.01***	1.64 ± 0.21*	0.88 ± 0.14	0.75 ± 0.18**
PS2APP	0.33 ± 0.04***	0.26 ± 0.03*	0.31 ± 0.02***	1.5 ± 0.45*	0.62 ± 0.12	0.88 ± 0.15**
Astrocytes						
wt	0.62 ± 0.06	0.65 ± 0.05	0.56 ± 0.05	2.65 ± 0.42	2.77 ± 0.38	2.33 ± 0.38
PS2-N141I	0.41 ± 0.03***	0.47 ± 0.04***	0.33 ± 0.04*	2.03 ± 0.32	2.22 ± 0.57	1.13 ± 0.27
PS2APP	0.38 ± 0.03***	0.34 ± 0.04***	0.41 ± 0.07	1.66 ± 0.3	1.44 ± 0.26	1.68 ± 0.46

Data are expressed as mean ± SEM of 8–11 slices from three different animals per genotype. Inh (5 μM NBQX, 25 μM D-AP5, 1 μM TTX). One-way ANOVA s followed by Tukey's HSD test show that values are statistically different from wt, *P < 0.05, **P < 0.01, ***P < 0.001.

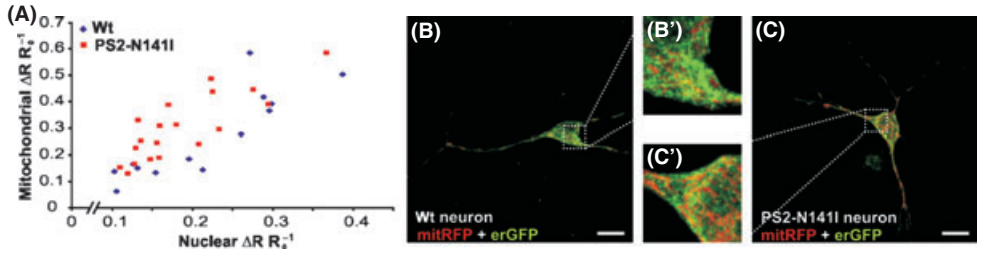

Fig. 5 Increased endoplasmic reticulum (ER)–mitochondria Ca^{2+} cross talk and colocalization in presenilin-2 (PS2)-N141I neurons. (A) Wt (blue squares) or PS2-N141I (red diamonds) neurons co-expressing H2BD1cpv and 4mtD1cpv were bathed in mKRB and exposed to mixed stimuli (500 μM CCH, 100 μM glutamate, 100 μM ATP) 2 min after addition of EGTA (2.5 mM). The peak of mitochondrial [Ca^{2+}] ($\Delta R/R_0$) is plotted as a function of the corresponding peak in the nuclear (cytosolic) [Ca^{2+}] ($\Delta R/R_0$). Only data from wt and tg neurons with comparable nuclear peaks ($0.1 < \Delta R/R_0 < 0.4$) are shown. (B,C) ER–mitochondria juxtapositions were visualized by confocal microscopy in wt (B) and PS2-N141I (C) neurons expressing mitRFP (red) and erGFP (green); yellow pixels indicate close proximity between the two organelles. Scale bar: 10 μm. (B′, C′) Enlarged details.

et al., 2009). Mitochondrial dysfunctions (either metabolic, structural or genetic) have widely been documented in aging and AD (Swerdlow *et al.*, 2010); moreover, endogenous as well as mutant PS2, but not PS1, have recently been shown to favor the physical and functional connection between these two organelles (Zampese *et al.*, 2011). We first investigated the energetic state of isolated brain mitochondria from three-month-old PS2APP mice compared with wt mice. No differences were found in classical parameters, *for example,* respiration rate (state 3 and state 4) and Ca^{2+} retention capacities (Fig. S4), indicating that, at this age, mitochondria from even double tg mice are not appreciably compromised. Of note, no difference in membrane potential, as measured by tetramethyl rhodamine methyl ester in intact cells, was observed in primary cortical neurons from tg and wt mice (not shown).

The mitochondria-ER Ca^{2+} cross talk was then investigated in wt and single tg neurons expressing a cameleon probe targeted to the mitochondrial matrix (4mtD1cpv) (Palmer *et al.*, 2006). To simultaneously follow cytosolic Ca^{2+} rises in the very same cell, the neurons were cotransfected with a nuclear Ca^{2+} probe (H2BD1cpv) (Giacomello *et al.*, 2010). The nucleoplasmic Ca^{2+} concentration very rapidly equilibrates with the cytosol, while its signal can be easily distinguished from the mitochondrial one (Zampese *et al.*, 2011). Single neurons coexpressing both probes were imaged for Ca^{2+} experiments and challenged with a mixture of IP_3-generating stimuli (500 μM CCH, 100 μM glutamate and 100 μM ATP) after Ca^{2+} chelation by EGTA (2.5 mM) addition. Upon stimulation, the nuclear Ca^{2+} rises ($nuc\Delta R/R_0$) of responding neurons ranged from 0.08 to 0.86 and from 0.04 to 0.37 for wt and single tg neurons, respectively, with a 50 ± 5.1% reduction in the average value of tg neurons ($nuc\Delta R/R_0$: 0.32 ± 0.05, $n = 22$ and 0.16 ± 0.02, $n = 25$ mean ± SEM for wt and tg mice, respectively, $P < 0.01$), in agreement with the reduced Ca^{2+} content of intracellular stores, as measured by Fura-2. However, for similar nuclear Ca^{2+} rises in the range of 0.1–0.4 $nuc\Delta R/R_0$, the mitochondrial responses of tg neurons were larger than those obtained in controls (Fig. 5A). Thus, the ratio between $mit\Delta R/R_0$ and $nuc\Delta R/R_0$ values, measured within this interval, was significantly higher in tg neurons with respect to wt ones (1.18 ± 0.11, $n = 13$ and 1.63 ± 0.1, $n = 19$ for wt and tg neurons, respectively; mean ± SEM, $P < 0.01$). Furthermore, with respect to controls, a closer apposition between ER and mitochondria was observed in single tg neurons transiently expressing ER- and mitochondria-targeted GFP/RFP variants (Fig 5B,C). The Manders coefficient for signal colocalization, calculated on Z stacks, was 0.43 ± 0.01 for wt neurons ($n = 40$) and 0.48 ± 0.01 for PS2-N141I neurons ($n = 50$), with a significant increase (11 ± 0.02%, $P < 0.01$) in the latter. Similar results were obtained in neurons from double tg mice (not shown). Consistently, after *in vivo* fixation, followed by immunolabelling with antibodies against mitochondrial (CoxIV) and ER (Grp78-Bip) markers, similar findings were obtained in both tg mice (Fig. S5A). Compared with controls, the Manders coefficient of signal colocalization and the area of close apposition between the two organelles were larger in tg mice of 12–15 days (11 ± 3.8% and 14 ± 4.3% increase in single and double tg mice, respectively; mean ± SEM, $n = 50$, three mice for each genotype, $P < 0.05$; Fig. S5B,C).

Neuronal Ca^{2+} excitability

In brain slices or cultured neurons, spontaneous Ca^{2+} spikes are very rare. However, such spontaneous spike activity can be drastically increased in frequency upon removal of GABA-A inhibition. In particular, cultured neurons show numerous synchronous Ca^{2+} spikes upon addition of the GABA-A antagonist picrotoxin (PTX, 50 μM). In both tg neurons, the frequency, but not the peak amplitude, of synchronous Ca^{2+} spikes in response to PTX was significantly increased (about 60%), compared with wt neurons (Fig. 6). In the three genotypes, the pattern of synchronous Ca^{2+} activity was not modified by 1 h pretreatment with either thapsigargin (1 μM) or ryanodine (50 μM) (not shown). These Ca^{2+} spikes were generated by glutamate release because they were fully abolished by the AMPA receptor antagonist NBQX (5 μM) (data not shown), while peak amplitude and duration of Ca^{2+} spikes were strongly reduced by the noncompetitive NMDA receptor antagonist MK-801 (10 μM) (Fig. S6A). Furthermore, these Ca^{2+} oscillations were inhibited by blocking presynaptic Ca^{2+} channels through acute application of ω-agatoxin IVA (0.2 μM), a blocker of P/Q type Ca^{2+} channels (Fig. S6B).

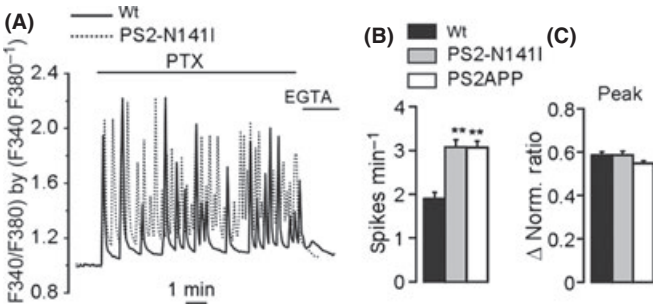

Fig. 6. Increased Ca^{2+} excitability in tg neurons. Synchronous Ca^{2+} spikes were induced in neurons at 17-19 DIV by a 10-min perfusion with PTX (50 μM) in mKRB. (A) Representative traces of wt and presenilin-2 (PS2)-N141I neurons. Bars show the average number of Ca^{2+} spikes per min (B) and peak amplitude calculated over the entire period (C) for the three genotypes. Values are expressed as mean ± SEM ($n = 19$ wt; $n = 21$ PS2-N141I; $n = 18$ PS2APP).

In acute brain slices, perfusion of Mg^{2+} -free ACSF supplemented with PTX (50 μM) leads to interictal like activity in neurons of the CA1 region (Gomez-Gonzalo *et al.*, 2010). The frequency of the interictal discharges, measured as Ca^{2+} transients, was doubled in both tg slices compared with wt ones (mean ± SEM spikes per min: 1.26 ± 0.24 $n = 10$; 2.34 ± 0.42, $n = 19$ $P < 0.05$; 2.94 ± 0.60, $n = 17$, $P < 0.05$, respectively, in wt, PS2-N141I and PS2APP slices from 5 to 6 mice per genotype), a finding consistent with the result obtained in cultured neurons.

It should be stressed that all the Ca^{2+} defects described previously do not result in overt neuronal toxicity. In particular, when the apoptotic index of neuronal cultures was measured at rest and in the presence of stressors acting through ER Ca^{2+} release, such as hydrogen peroxide or ceramide – added either alone or together with DHPG – a trend to a higher vulnerability, albeit not significant, was found in neurons from the tg animals (not shown).

Role of Aβ

Both FAD-linked PS1 and PS2 mutations have been associated with altered Aβ peptide generation, resulting in increased Aβ42/Aβ40 ratios (Borchelt *et al.*, 1996). The PS2APP mice were reported to produce similar levels of both peptides, whose content increases from 0.1 to 10 ng mg^{-1} brain (wet tissue) from the third to the sixth month of age, that is, when the first amyloid deposits are detectable in the cortex and hippocampus (Richards *et al.*, 2003; Ozmen *et al.*, 2009). Conversely, the PS2-N141I line (not previously tested) is expected to have a much reduced total Aβ load compared with the PS2APP line. The question thus arises as to whether the effects on the ER/mitochondria Ca^{2+} handling and excitability depend primarily on the mutant PS2 or on the Aβ production. We thus measured the amount of total Aβ load in the brain of tg mice at 2 weeks of age (i.e., when brain slices were prepared for Ca^{2+} measurements). Compared with controls, the Aβ42 levels were 4 and 40 times larger in single and double tg mice, respectively (Fig. S7A), ranging from 4 to 40 pg mg^{-1} wet tissue (1–10 pmol g^{-1}), at least 10^2 times smaller than that found at 6 months of age in amyloid seeding brains (Ozmen *et al.*, 2009). Moreover, while in double tg mouse brains the Aβ42/Aβ40 ratio was close to one, as expected (Ozmen *et al.*, 2009), in the single tg animals it was higher than in wt, but well below one (Fig. S7B).

Discussion

The mechanisms underlying neuronal dysfunction in the so-called sporadic AD cases is still debated and clearly involve genetic predisposition, environmental components and, primarily, age factors. A major limitation for research on sporadic AD is the lack of suitable animal models. In contrast, major breakthroughs in understanding the disease pathogenesis have been obtained from studies based on the rare FAD cases linked to autosomal dominant mutations in APP and PSs. Although no consensus has yet been reached, many studies suggest that alterations of neuronal Ca^{2+} homeostasis characterize normal brain aging as well as several neurodegenerative disorders; though, it remains unknown whether such modifications of Ca^{2+} handling are a late consequence or a primary cause of neuronal dysfunction (Toescu & Verkhratsky, 2007; Toescu & Vreugdenhil, 2010). Of note, the fact that PSs play a regulatory role in ER Ca^{2+} uptake and release has received much attention in the AD field (Thinakaran & Sisodia, 2006). Evidence accumulated for many years has established a strong relationship between FAD-linked PS mutations and dysregulation of intracellular Ca^{2+} homeostasis (Camandola & Mattson, 2011). The issue is still quite confusing at the moment, as contradictory results have been obtained by different laboratories using different approaches. A key problem for solving these discrepancies is the choice of the experimental models. The most relevant ones are probably tg mice, as they express mutant PS at *quasi*-normal levels. Moreover, they can be analyzed before the onset of any neurological defect, thus clarifying whether alterations of Ca^{2+} signaling anticipate, are concomitant or follow the other pathological signs, such as increase in Aβ load and plaque deposition, synapse restructuring, astrogliosis and neuronal loss.

Here, we have taken advantage of two tg mouse lines: PS2.30H, expressing only the PS2-N141I, and B6.152H, expressing also the APPswe. Of note, both lines have PS2 levels quite similar to those found in wt animals. We have studied the characteristics of the Ca^{2+} response both in primary neuronal cultures, obtained from neonatal pups, and in acute brain slices from 2-week-old mice. At this age, total Aβ levels are still very low, but already detectable and higher in the double tg line. As to the choice of tg mice carrying a FAD-linked PS2 mutant, rather than one of the most commonly employed PS1 mutant, it is based on two considerations: (i) we have extensively characterized a number of PS2 mutants in cell lines or human FAD fibroblasts with consistent and reproducible findings; (ii) the phenotypic characteristics of FAD patients bearing mutations in PS1 or PS2 are practically indistinguishable, but for milder aggressiveness and later onset, usually, in PS2-linked FAD cases. Thus, if an alteration in Ca^{2+} homeostasis is causal in the disease, the prediction is that it should be, at least qualitatively, similar for both PS1 and PS2 mutants.

The data here presented demonstrate unambiguously that what was observed in transiently expressing cell lines and neurons does not depend on artefacts of protein over-expression, but represents an intrinsic specific action of FAD-mutant PS2 in modulating Ca^{2+} handling. In particular, the reduction in ER Ca^{2+} content (as assayed indirectly from the cytosol by discharging the organelle Ca^{2+} with ionomycin or IP_3-generating agonists, and directly from inside the organelle by measuring Ca^{2+} levels with a specific ER probe) is of similar entity to that previously estimated in cell lines transiently over-expressing the same mutant PS2 at high levels (Zatti *et al.*, 2006; Zampese *et al.*, 2011). Most important, the same extent of reduction was found in neurons of acute brain slices. Thus, the altered Ca^{2+} handling caused by mutant PS2 revealed in cultured cells is maintained in the more physiologically relevant *in situ* model. It needs also stressing that a reduction in IP_3-mobilizable Ca^{2+} in slices from both tg mice is observed not only in neurons, but also in astrocytes. Since mutant APP is expressed only in neurons while PS2-N141I in both cell types, the fact that astrocytes show similarly altered Ca^{2+} dynamics in the double tg mice further suggests that the mutant PS2 is the only culprit. Last, but not least, the similarity (qualitatively and quantitatively) between Ca^{2+} dysregulation in the two tg mouse lines confirms that expression of mutant APP, *per se*, has no primary effect on the store Ca^{2+} content, at least at 2 weeks of age (Stieren *et al.*, 2010). Taken together these findings point out to an 'all or none' effect of mutant PS2 that may be related to the amount of its possible interacting partners.

In spite of the reduced store Ca^{2+} content, Ca^{2+} release induced by caffeine in cultured tg neurons was increased, a finding common not only to other AD mouse models based on different FAD-linked PS mutations (Chan *et al.*, 2000; Smith *et al.*, 2005), but also a possible biomarker of aging neurons (Thibault *et al.*, 2007; Toescu & Verkhratsky, 2007; Toescu & Vreugdenhil, 2010). Most likely this larger response to caffeine depends on the increased level of Ry-Rs in brains and cultured neurons from both tg mice, as compared to wt. Much to our surprise, no rapid Ca^{2+} mobilization could be triggered in slices by caffeine, when applied either as a puff or by perfusion. However, the drug was effective on Ry-Rs, as it induced a complete emptying of Ca^{2+} stores after a prolonged incubation, while blocking Ca^{2+} spikes with TTX. Taken together, these

data suggest that the penetration of caffeine inside the cells is too slow to rapidly reach a sufficiently high concentration capable of triggering fast Ca^{2+} mobilization via Ry-Rs, but it is enough to slowly and completely discharge the ER Ca^{2+} content.

We have recently reported that over-expression of mutant PS2 increases the contact sites between ER and mitochondria in cell lines, as well as in primary rat cortical neurons (Zampese *et al.*, 2011). From the functional point of view, this structural change is also responsible of an increased efficiency of Ca^{2+} uptake by mitochondria because of an increased number of Ca^{2+} microdomains (formed at the outer mitochondrial membrane upon ER Ca^{2+} release) (Zampese *et al.*, 2011). ER–mitochondria contact sites were significantly increased also in tg neurons of primary cultures and brain slices, as compared to wt neurons. As to ER–mitochondria Ca^{2+} transfer, because of the strong reduction in the ER Ca^{2+} content in PS2-N141I neurons, the maximal nuclear (cytosolic) and mitochondrial increases were observed in the wt neurons. However, if one considers only wt and tg neurons with comparable nuclear (cytosolic) responses, the rise in mitochondrial Ca^{2+} was significantly larger in the latter cells.

An extensive body of literature suggests that Aβ peptides may affect Ca^{2+} homeostasis (Thinakaran & Sisodia, 2006). We here show that, in the brains of 2-week-old tg mice, the level of total Aβ42 and the Aβ42/Aβ40 ratio were rather low but yet substantially higher in the PS2APP mice. Given that the reduction in the store Ca^{2+} content, the increase in Ry-R expression and the augmented ER–mitochondria cross talk, are all very similar in the two tg lines, the simplest explanation is that Aβ42 and Aβ40 peptides do not play a major role in these processes. It may be argued that the Aβ increase observed in PS2 tg mice is sufficient to cause the Ca^{2+} alterations we observed and that the additional larger Aβ rise observed in the PS2APP mice has no additional effect. We believe that this possibility is unlikely, because qualitatively and quantitatively similar effects on Ca^{2+} stores and ER–mitochondria coupling were found in cell lines and neurons expressing the loss-of-function PS2-D366A (Brunello *et al.*, 2009; Zampese *et al.*, 2011). We cannot exclude, on the contrary, that the increase in Ca^{2+} spikes upon GABA-A receptor blockade is already maximal at the very low levels of Aβ42 found in single tg mice. Similarly, it is likely, [and actually important evidence has recently been obtained along these lines (Busche *et al.*, 2012)], that toxic Aβ peptides, at the higher levels reached in older mice, will *per se* cause modifications in neuronal Ca^{2+} homeostasis. Altogether, our data are in agreement with the idea that increased neuronal Ca^{2+} excitability is a precocious event shared by both AD and brain aging (Gleichmann *et al.*, 2012).

The final and most important question is whether the altered Ca^{2+} homeostasis – here revealed in neurons (and astrocytes) from tg mice bearing the PS2-N141I – has a role in the pathogenesis of the disease. Our data clearly demonstrate that the so-called 'Ca^{2+} overload' mechanism (La Ferla, 2002) is untenable in FAD caused by mutations in PS2. Along the same line, a thorough, very recent, study by Foskett and coworkers reveals that a number of PS1 mutations neither decrease nor augment ER Ca^{2+} levels (Shilling *et al.*, 2012). In support of data previously obtained in cells and neurons over-expressing FAD-linked PS2 mutants, we here show, both in primary cultures and *in situ*, that neurons from tg mice carrying the PS2-N141I mutation have a lower ER Ca^{2+} content, but the number of contacts sites between mitochondria and ER is increased. In contrast, PS1 mutations do not cause a major change of ER Ca^{2+} levels and do not modify ER–mitochondria tethering (Zatti *et al.*, 2006; Zampese *et al.*, 2011; Shilling *et al.*, 2012). Both FAD-PS1 and PS2 mutations increase the expression of Ry-Rs and the sensitivity of IP_3-Rs to IP_3 (Cheung *et al.*, 2008). A revised version of the Ca^{2+} overload hypothesis that takes into consideration all the above data would rather point to an increased transfer of Ca^{2+} from the ER to the mitochondria as a causative event: with PS2 mutations, the increased IP_3-R sensitivity and Ry-R number, coupled to a higher ER–mitochondria juxtaposition, may lead to a larger Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter, despite the reduced overall ER Ca^{2+} content. In the long run, this chronic over-transfer of Ca^{2+} to mitochondria may eventually lead to mitochondrial functional impairment and neuronal damage. In this context, the increased Ca^{2+} excitability of tg neurons, here revealed, may represent a further stressor. In the case of PS1 mutations, the ER Ca^{2+} level is unperturbed (or possibly increased) and the ER–mitochondria tethering is normal, but the IP_3-R sensitivity and the Ry-R number are both increased as in mutated PS2 models. In the end, in neurons bearing PS1 mutations, the effect on ER–mitochondria Ca^{2+} transfer may be similarly enhanced. In addition, the possibility should be considered that the Ca^{2+} handling alterations here reported may be more directly correlated with modifications in synaptic functions rather than to overt cell death. Indeed, mitochondrial Ca^{2+} dysregulation has been linked to altered synaptic plasticity in normal aging (Toescu & Verkhratsky, 2004) and, possibly, to the synaptic dysfunctions precociously observed in different AD mouse models. Noteworthy PS1 has recently been shown to influence dendritic spine plasticity in an *in-vivo* model, independently of its γ-secretase activity, but possibly linked to an effect on Ca^{2+} homeostasis (Jung *et al.*, 2011).

Our proposed model takes into account findings by different laboratories and provides a rationale that accommodates divergent data into a unifying hypothesis centred on mitochondrial Ca^{2+} toxicity. How altered ER/mitochondria Ca^{2+} cross talk then modifies synaptic plasticity and causes early dysfunctions is a matter of intense study. Briefly, we can first cite altered mitochondria trafficking at the synaptic level and, consequently, reduced ATP supply and Ca^{2+} buffering capacity at the site of highest demand, all conditions which can be worsened by the increased excitability.

Obviously, the model proposed is clearly oversimplified as it does not consider other key pathogenic factors that are essential in AD, *that is*, Aβ load and plaques, hyperphosphorylated tau and astrogliosis. However, the fact that, in different AD mouse models, the presence of PS mutations accelerates the onset of neuronal dysfunctions and cognitive defects, reinforces the idea that subtle changes in Ca^{2+} handling, owing to PS mutants, render the neurons more vulnerable to other insults, such as Aβ oligomers, reactive oxygen species and excitotoxicity.

Experimental procedures

Animal handling and care

The transgenic mouse lines PS2.30H and B6.152H were kindly donated by Dr. L. Ozmen (F. Hoffmann-La Roche Ltd, Basel, Switzerland) (Richards *et al.*, 2003; Ozmen *et al.*, 2009; Rhein *et al.*, 2009). Both lines have the background strain of C57BL/6 mice, which were used as wt controls and purchased from Charles River (Lecco, Italy). All procedures were carried out in strict adherence to the Italian regulations on animal protection and care and with the explicit approval of the local veterinary authority (CEASA Nr 56880).

Primary neuronal cultures

Primary neuronal cultures were obtained from cortices dissected from 0 to 1 day newborn mice as previously described (Zatti *et al.*, 2006). Cells were seeded on poly-L-lysine (100 μg mL^{-1}) coated coverslips at a density of 300.000 cells cm^{-2} in MEM Gibco containing glucose (20 mM), L-glutamine (0.5 mM), N2 supplement (1%), B27 supplement (0.5%), biotin

(3.6 μM), pyruvic acid (1 mM), penicillin (25 μg mL^{-1}), streptomycin (25 μg mL^{-1}), neomycin (50 μg mL^{-1}) and horse serum (10%). After 24 h plating, the complete MEM was replaced with serum and antibiotic free Neurobasal medium containing B27 (2%) and L-glutamine (2 mM), unless otherwise stated. Fresh medium was added (1/5 of total volume) every 4th day.

Ca^{2+} measurements in primary neuronal cultures

Cytosolic and organelle (mitochondria, nuclear and ER) Ca^{2+} levels were monitored by different approaches (see Data S1).

Data analysis

Data were analyzed using Origin 7.5 SR6 (OriginLab Corporation, Northampton, MA, USA) and Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Traces are averages of 40–60 cells and are representative of 20–40 independent experiments. Values are expressed as mean ± SEM (n = number of independent experiments). Statistical significance was evaluated by unpaired two-tailed Student's t-test. Analysis of the differences between categories was carried out by one-way ANOVA followed by Tukey's HSD (Honestly Significantly Different) multiple comparison tests with a confidence interval of 95%, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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Conflict of interest

None.

Author contributions

P.P., C.F and T.P designed the experiments and wrote the manuscript. M.J.K. and L.C. helped in writing the manuscript and performed the experiments with neuronal cultures and brain slices, respectively. E.Z., C.L. and A.W. performed the experiments with the recombinant probes. The biochemical and histochemical analyses of mouse brains and cultures were carried out respectively by L.C. and C.L.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1 Chemicals and reagents, Ca^{2+} measurements in primary neuronal cultures, Ca^{2+} measurements in acute brain slices, Colocalization analysis, ELISA, Western blots, Mitochondria isolation, respiration and Ca^{2+} retention

Fig. S1 KCl treatment of neuronal cultures.

Fig. S2 Astrocyte responses to DHPG in acute brain slices.

Fig. S3 Ry-R2 is up-regulated in tg mice.

Fig. S4 Bioenergetics of isolated mitochondria.

Fig. S5 ER–mitochondria interactions in hippocampal slices of wt and tg mice.

Fig. S6 PTX-induced Ca^{2+} spikes are due to released glutamate.

Fig. S7 Aβ levels in mouse brains.

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