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# Capacitive Calcium Entry Is Directly Attenuated by Mutant Presenilin-1, Independent of the Expression of the Amyloid Precursor Protein*

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**Jochen Herms‡§, Ilka Schneider‡§, Ilse Dewachter§¶‖, Nathalie Caluwaerts¶, Hans Kretzschmar‡, and Fred Van Leuven¶****

*From the ‡Department of Neuropathology, Ludwig-Maximilians-Universität, 81377 Munich, Germany and the ¶Experimental Genetics Group, Department of Human Genetics, B-3000 Leuven, Belgium*

**Mutant presenilin-1 (PS1) increases amyloid peptide production, attenuates capacitative calcium entry (CCE), and augments calcium release from the endoplasmatic reticulum (ER). Here we measured the intracellular free $Ca^{2+}$ concentration in hippocampal neurons from six different combinations of transgenic and gene-ablated mice to demonstrate that mutant PS1 attenuated CCE directly, independent of the expression of the amyloid precursor protein (APP). On the other hand, increased $Ca^{2+}$ release from the ER in mutant PS1 neurons, as induced by thapsigargin, was clearly dependent on the presence of APP and its processing by PS1, *i.e.* on the generation of the amyloid peptides and the APP C99 fragments. This observation was corroborated by the thapsigargin-induced increase in cytosolic $[Ca^{2+}]_i$ in PS1 deficient neurons, which accumulate C99 fragments due to deficient γ-secretase activity. Moreover, co-expression of mutant APP[V717I] in PS1-deficient neurons further increased the apparent size of the ER calcium stores in parallel with increasing levels of the APP processing products. We conclude that mutant PS1 deregulates neuronal calcium homeostasis by two different actions: (i) direct attenuation of CCE at the cell-surface independent of APP; and (ii) indirect increase of ER-calcium stores via processing of APP and generation of amyloid peptides and C99 fragments.**

The formation of amyloid peptides (Aβ40/42) by proteolytic processing of the amyloid precursor protein (APP)[1] is proposed to be central to the pathology in Alzheimer's disease (AD) (1). The amyloid peptides are neurotoxic and alter calcium ion homeostasis (for review, see Ref. 2). Most familial forms of AD are caused by a mutation in presenilin-1 (PS1) (for review, see 3). Mutant PS1 increases the production of amyloid peptides by γ-secretase cleavage of APP, an activity for which PS1 is essential (4). In addition, however, mutant PS1 deregulates intracellular calcium homeostasis by increasing the apparent intracellular pools of $Ca^{2+}$ and decreasing capacitative calcium entry (CCE) (Refs. 5–7, and references therein). CCE is a refill mechanism allowing entry of extracellular calcium ions through plasma membrane channels that are tightly regulated by and even physically linked to intracellular stores (for review, see Ref. 8). Reduced CCE in neurons from mutant PS1 transgenic mice (5, 6) appeared to increase the levels of Aβ42 in cultures, whereas exogenously added peptide had no effect on CCE (6). The mechanisms by which mutant PS1 deregulates neuronal calcium homeostasis are not known, but the relation of a perturbed flux of calcium ions to the alteration in APP processing, or *vice versa*, is debated or questioned as an essential element in the pathogenic processes (5, 6, 7 and references therein).

Here, we have analyzed cellular mechanisms in neurons that were derived from six different types of single and multiple transgenic or gene-ablated mice. We analyzed, for the first time, neurons that express a mutant PS1 on a wild-type and an APP-deficient background, and in addition we analyzed the effect of a mutant APP expressed in wild-type and in PS1-deficient neurons. First and foremost, we demonstrate here that CCE is normal in the absence of APP, whereas APP deficiency did not correct the attenuated CCE provoked by mutant PS1. These findings implicate PS1 directly in CCE, independent of its activity as γ-secretase on APP. We further measured and compared the thapsigargin-induced increase in $[Ca^{2+}]_i$ as a measure of ER calcium stores in neurons from the six different transgenic mouse strains, including mice with a neuronal deficiency in PS1, *i.e.* PS1 (n−/−) crossed with APP[V717I] mice (9). The results demonstrate that the increase in ER calcium stores in neurons is due to increased processing of APP into amyloid peptides and C99 fragments.

## EXPERIMENTAL PROCEDURES

*Strains of Transgenic Mice*—The parent APP[V717I], PS1[wt], and PS1[A246E] transgenic mice (7, 10, 11, 12) and APP−/− mice (13) were described previously. Viable adult mice with a specific neuronal deficiency in PS1, denoted PS1(n−/−), were generated by crossing mice with a floxed PS1 gene with mice expressing Cre recombinase under control of the mouse *thy1* gene promoter (9). PS1(n−/−) mice are viable and fertile and have normal brain morphology and normal behavior (9). They were crossed with APP[V717I] transgenic mice (10) to demonstrate effective inhibition of amyloid peptide and plaque formation up to the age of 18 months (9).

*Neuronal Cell Cultures*—Hippocampi from newborn mice were dissociated with papain (25 units/ml, with cysteine, 2 mg) in Dulbecco's modified Eagle's medium for 60 min at 37 °C before transfer to Dulbec-

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§ These authors contributed equally to this work.
‖ Postdoctoral fellow at Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.
** To whom correspondence should be addressed: Experimental Genetics Group, Dept. of Human Genetics, KULeuven-Campus Gasthuisberg ON_06, B-3000 Leuven Belgium. Tel.: 32-16-3458-88; Fax: 32-16-3458-71; E-mail: fredvl@med.kuleuven.ac.be.

[1] The abbreviations used are: APP, amyloid precursor protein; AD, Alzheimer's disease; PS1, presenilin-1; CCE, capacitative calcium entry; DFI, digital fluorescence images; AICD, APP intracellular domain.



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co's modified Eagle's medium/fetal calf serum (5%), $N_2$ supplement, glutamine, and antibiotics. Cells were triturated and, after attachment on wild-type astrocyte feeder layers, the medium was changed to neurobasal medium with B27 supplement (Invitrogen). Neurons were cultured for 19–21 days, and experiments were performed in Locke's buffer, *i.e.* 154 mM NaCl, 5.6 mM KCl, 2.3 $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES (pH 7.2). Dissociation of neurons from adult mouse hippocampi was as described (7).

*Calcium Imaging*—$[Ca^{2+}]_i$ measurements in hippocampal neurons from newborn mice and dissociated neurons from adult mice were performed using the indicator fura-2-acetoxymethyl ester (fura-2/AM; Molecular Probes, Göttingen, Germany). Fura-2/AM was solubilized in Dulbecco's modified Eagle's medium with pluronic acid (0,08%) in HBSS (145 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$) containing bovine serum albumin (1%). Cells on cover slips were loaded at 37 °C for 30 min and transferred after 30 min of washing time to a microchamber on the stage of an upright microscope (BX50 WI, Olympus) to be viewed under visible and UV light using a 60× water immersion objective. Approximate intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) were calculated from the ratio of a fura-2 emission evoked by 340-and 380-nm light from a 75-W argon lamp using a digital imaging system (Till Photonics, Munich, Germany). Measurements were acquired at 1–3 s intervals at both excitation wavelengths (340 and 380 nm). Digital fluorescence images were constructed and displayed as pseudo-color images and subsequently analyzed (Vision software, Till Photonics, Munich, Germany). $[Ca^{2+}]_i$ was calculated for each pixel in the frame with fluorescence intensities over a defined threshold (14). Each experiment was performed on at least four cultures after 19–21 days in culture and prepared from different mice. Experiments were performed at room temperature, and drugs were applied by bath superfusion (2 ml/min). For $Ca^{2+}$-free experiments the same buffer was used, but $CaCl_2$ was omitted and 50 $\mu$M EGTA was added.

$[Ca^{2+}]_i$ measurements in hippocampal slices were performed using patch clamp methodology on pyramidal cells in thin slices following standard procedures (15, 16). Briefly, the brain from an adult mouse (6–8 months) was dissected, and the hippocampus was rapidly isolated and kept in ice-cold bicarbonate-buffered saline solution (125 mM NaCl, 1.25 mM KCl, 1.25 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 16 mM glucose). Transverse vibratome slices (150 $\mu$m) (16, 17) were maintained for at least 1 h at 34 °C in the same buffered saline solution with continuous oxygenation (95% $O_2$, 5% $CO_2$). Slices were transferred to the recording chamber and superfused with the same solution at room temperature. Pyramidal cells were selected under the microscope using a 60× water-immersion objective. Electrodes pulled from borosilicate glass capillaries were filled with loading solution containing 125 $\mu$M Fura-2, 140 mM $CsCl_2$, 2 mM $MgCl_2$, 4 mM ATP, 0.4 mM GTP, and 10 mM HEPES (pH 7.3) (pipette resistance 2–3 MV). Whole-cell recordings were performed with a patch clamp amplifier (EPC-9, HEKA Electronic, Lambrecht, Germany). Cells were voltage clamped at a slightly depolarizing holding potential (−60 mV). Changes in $[Ca^{2+}]_i$ were monitored using a digital imaging system as described above. Following establishment of the whole-cell configuration, loading of the cell with fura-2 was monitored until equilibration between pipette and proximal dendrite as indicated by stable maximal intensity signals at 340 nm. Consecutive paired exposures to 340 and 380 nm were used to construct background-corrected digital fluorescence images. The gain of the intensified video CCD camera was set at values that optimized detection of signals from the cell soma, and $[Ca^{2+}]_i$ was calculated (14). The calibration constants $K_{eff}$ (effective binding constant) and $R_{min}$, *i.e.* fluorescence ratio at zero $Ca^{2+}$, were obtained from *in vivo* calibration experiments as described (18, 19). These parameters varied slightly in time, depending on several factors such as aging of the UV lamp used for excitation, but typical values for $K_{eff}$, $R_{min}$, and $R_{max}$ were 420, 0.4 and 1.4 nM, respectively. All patch clamp measurements were performed at room temperature.

## RESULTS

*Mutant PS1 Attenuates CCE Independent of Expression of APP*—CCE was measured in neurons from non-transgenic mice in parallel with neurons from transgenic mice expressing human mutant PS1[A246E] or human wild-type PS1 as an extra control. CCE was induced by a published protocol, *i.e.* after preincubation in $Ca^{2+}$-free media containing cyclopiazonic acid to deplete the ER $Ca^{2+}$ stores, and the neurons were replenished with $Ca^{2+}$-containing media (1.8 mM) (6, 20). CCE was strongly decreased in PS1[A246E] neurons (Fig. 1, *A* and *B*) and, as an extra control, we observed that under the same conditions SKF-96365 inhibited CCE in wild-type neurons (Fig. 1, *C* and *D*).

We then observed that CCE was normal in neurons derived from APP−/− mice (Fig. 1, *C* and *D*), excluding endogenous APP as a direct or indirect mediator of the effect of mutant PS1. We further analyzed CCE in neurons from double transgenic mice expressing the mutant PS1 on an APP-deficient background, *i.e.* APP−/− × PS1[A246E]. It must be noted that the APP−/− × PS1[A246E] mice were normally viable and fertile and did not show phenotypic or histological abnormalities of the brain (results not shown). Most importantly, however, we observed that CCE in the APP−/− × PS1[A246E] neurons was very similar to that in PS1[A246E] neurons, *i.e.* in both CCE was significantly suppressed relative to non-transgenic or APP−/− neurons (Fig. 1, *E* and *F*).

The combined results demonstrated that mutant PS1 reduced neuronal CCE independent of the expression of APP, which in itself also had no negative effect on CCE. This is the first demonstration that attenuated CCE is a direct consequence of mutant PS1, whereas APP and its proteolytic processing are not involved in this process. It is therefore concluded that PS1 is not implicated in CCE as the alleged $\gamma$-secretase but via another inherent or associated activity.

*Mutant PS1 Increases ER Calcium Stores Only in the Presence of APP*—Because APP processing did not affect CCE, we examined whether the same would hold true for the ER calcium ion stores, which are increased by mutant PS1 (7, 9). The analysis was performed by measuring the response to thapsigargin in hippocampal neurons derived from six different transgenic mouse stains, *i.e.* transgenic mice with mutant PS1 and/or mutant APP and in combination with a deficiency in APP (13) or in PS1 (9) (Table I).

First and foremost we observed that, similar to CCE, the absence of APP did not markedly affect the extent or the kinetics of changes in $[Ca^{2+}]_i$ in cultured hippocampal neurons in response to thapsigargin (Fig. 2, *C* and *D*). On the other hand, and opposite to the effect on CCE, the absence of APP completely abolished the marked increase in $[Ca^{2+}]_i$ observed in mutant PS1 neurons (Fig. 2, *E* and *F*).

This demonstrated that the absence of APP did not negatively affect either neuronal CCE or the ER-based calcium ion stores. The results do reveal, however, that mutant PS1 can only increase ER-based calcium ions stores in the presence of APP, which evidently implicates APP itself or more likely its proteolytically derived fragments in this intracellular phenomenon.

*Deficiency of PS1 Also Causes Overfilling of ER Calcium Stores*—In dissociated hippocampal neurons expressing APP[V717I], the peak $[Ca^{2+}]_i$ response to thapsigargin was marginally increased relative to neurons from non-transgenic mice (Fig. 3, *A* and *B*). The same mutant APP[V717I] expressed in PS1-deficient neurons, however, dramatically increased the amplitude of the response to thapsigargin (Fig. 3, *C* and *D*), indicating that the absence of PS1 strongly augmented the effect of mutant APP. This was also evident in PS1(n−/−) neurons that react to thapsigargin by a robust and significant increase in cytoplasmic calcium ion levels, although less pronounced than APP[V717I] × PS1(n−/−) neurons (Fig. 3, *C* and *D*) (Table I).

These important observations were further corroborated in experiments with hippocampal slice preparations from the same strains of transgenic mice in which the fluorescent dye was loaded from the patch pipette (17). Brain sections have the unmistakable advantage that the neurons maintain their phys-

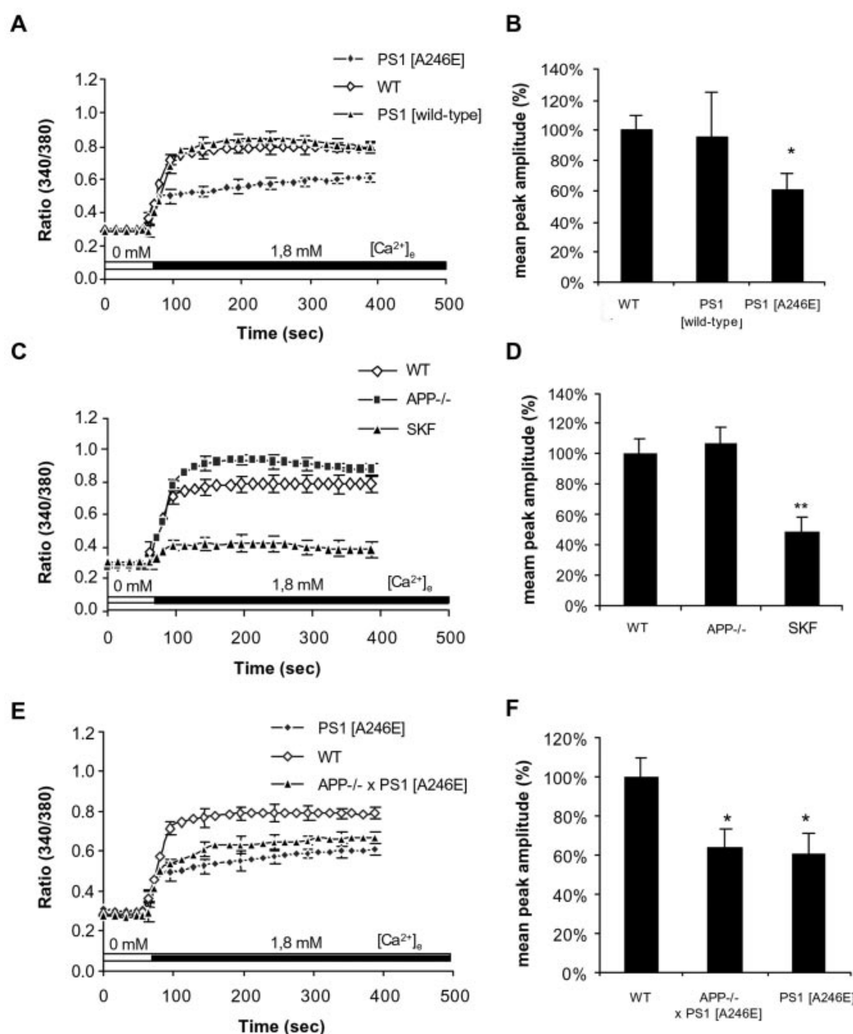


FIG. 1. **Attenuation of CCE by mutant PS1 is not dependent on expression of APP.** *A* and *B*, decreased CCE in hippocampal neurons from PS1[A246E] transgenic mice ($n$ = 30 cells) as opposed to normal CCE in neurons from non-transgenic mice and human wild-type (*WT*) PS1 transgenic mice (*, $p < 0.05$). *C* and *D*, cultured hippocampal neurons from non-transgenic and APP−/− mice have similar CCE, and CCE is inhibited by SKF-96365 (*SKF*; 100 $\mu$M). Data points are mean ± S.E. ($n$ = 40 cells) (**, $p <$ 0.001). *E* and *F*, decrease in CCE is similar in neurons from PS1[A246E] and APP−/− × PS1[A246E] mice (*, $p <$ 0.05). $[Ca^{2+}]_i$ was measured by ratiometric imaging of fura-2 in primary cultures of hippocampal neurons as illustrated in Fig. 2*A*.

TABLE I

*Overview of the six transgenic mouse strains analyzed with the most relevant characteristics*

In the top two rows, CCE and the thapsigargin response were analyzed in neuronal cell cultures, and hippocampal slice preparations were derived from the six single or combined transgenic mouse strains. The A$\beta$ and C99 levels refer to the total levels of amyloid peptides and C99 fragments of APP, respectively, in the brains of the transgenic mice as published (7–12). The symbols used to denote the (semi)-quantitative changes as discussed in the text are: =, not significantly different; ○, absent; N.D., not determined; + and −, increased or decreased, respectively, proportional with the number of signs.

| Factors | PS1[A246E] | APP−/− | PS1[A246E] × APP−/− | APP[V717I] | PS1(n−/−) | APP[V717I] × PS1(n−/−) |
|---|---|---|---|---|---|---|
| CCE | − | = | − | N.D. | N.D. | N.D. |
| Thapsigargin response | + | = | = | = | + | ++ |
| A$\beta$ level | + | ○ | ○ | ++ | = | = |
| C99 level | = | ○ | ○ | + | ++ | +++ |

iological context with many cell processes intact. The basal levels of $[Ca^{2+}]_i$ in neurons in the sections were lower than in isolated neurons but very similar for all genotypes analyzed (Fig. 4).

Most importantly, in response to thapsigargin, $[Ca^{2+}]_i$ was again most markedly enhanced in hippocampal neurons in slice preparations from PS1(n−/−) × APP[V717I] mice (Fig. 4, *B* and *C*) relative to APP[V717I] neurons, which showed a marginal increase (Fig. 4*C*). Remarkably again, in preparations from PS1(n−/−) mice the increase in $[Ca^{2+}]_i$ response to thapsigargin was significant (Fig. 4*C*), confirming the results obtained in cultured hippocampal neurons (Fig. 3).

The concordant results demonstrated that the deficiency of PS1 on its own did markedly affect the response to thapsigargin as an indication of the size of ER-based calcium ion stores. The responses were further augmented by coexpression of mutant APP. These data warrant the conclusion that PS1 affected the ER-based calcium ion stores indirectly and only by the intermediation of APP, which, considering the nature of these proteins and their known relationship, implicates the proteolytic processing products from APP as prime candidates (Table I) (9, 21).

## DISCUSSION

Although the normal physiological functions of both APP and PS1 and their intricate relationship are still unclear and heavily debated, the evidence that mutant presenilins generate more hydrophobic amyloid peptides is accepted (for reviews, see Refs. 22–24). The final step in the processing of APP and the generation of the amyloid peptides is $\gamma$-secretase cleavage,

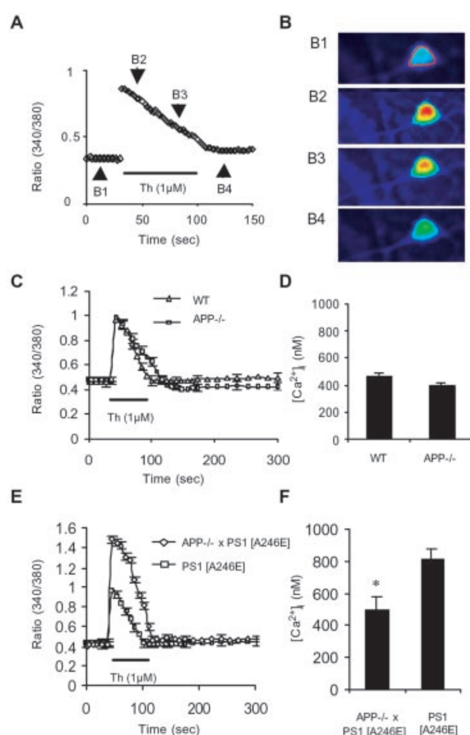


FIG. 2. **ER-calcium stores are normal in APP−/− neurons and increased in PS1[A246E] neurons.** *A* and *B*, a representative experiment of $[Ca^{2+}]_i$ measurements by ratiometric imaging of fura-2 in primary cultures of hippocampal neurons. The time course of fluorescence ratio (340/380 nm), with time points indicated (*panel A*), of digital fluorescence images (*panel B*) of a fura-2 loaded pyramidal cell is shown. The ratio signal in *panel A* was calculated over the area outlined in *section B1* of *panel B*. *C* and *D*, response to thapsigargin (*Th*) is similar in hippocampal neurons from wild-type mice (*WT*) and APP-deficient mice (APP−/−). *E* and *F*, correction by APP deficiency of the increased response of $[Ca^{2+}]_i$ in PS1[A246E] neurons triggered by thapsigargin. All data points represent mean fluorescence ratio ± S.E. ($n$ = 40–50 cells) (*, $p < 0.05$). Thapsigargin (1 μM) was applied as indicated by the *bars* under the tracings in *panels A*, *C*, and *E*.

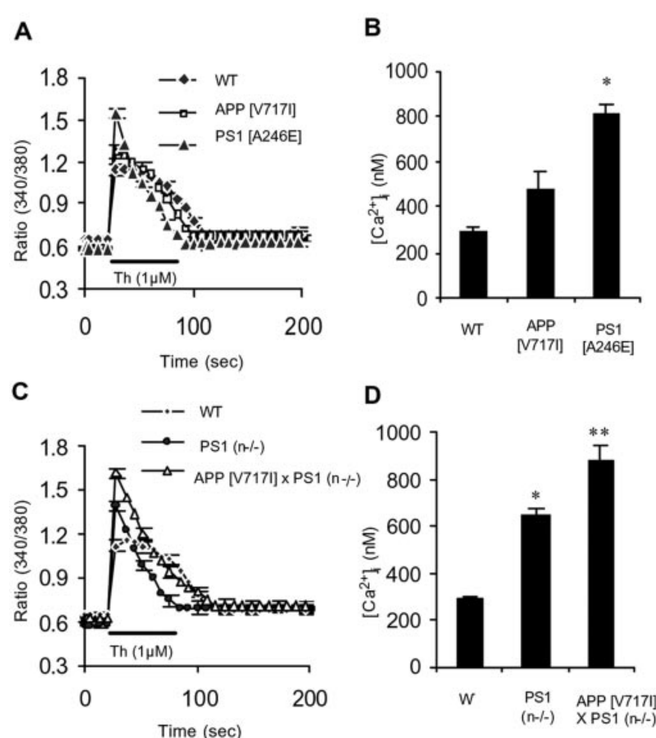


FIG. 3. **Increased ER-calcium stores in PS1(n−/−) and APP[V717I] × PS1(n−/−) neurons.** $[Ca^{2+}]_i$ was measured by ratiometric imaging of fura-2 (see Fig. 2 and "Experimental Procedures" for details). *A* and *B*, increased response to thapsigargin (*Th*) of $[Ca^{2+}]_i$ in PS1[A246E] neurons (*, $p < 0.05$) and the non-significant effect in APP[V717I] neurons. *C* and *D*, increased response to thapsigargin of $[Ca^{2+}]_i$ in PS1-deficient neurons (PS1(n−/−) (*, $p < 0.01$) is strongly accentuated by the expression of APP[V717I] (**, $p < 0.005$). Thapsigargin (1 μM) was applied as indicated by the *bars* under the tracings (*panels A* and *C*). Data points are mean values ± S.E. of 40–55 cells from at least three independent preparations.

for which PS1 is essential (4). The primary pathogenic changes in AD have been attributed to the amyloid peptides, and different mechanisms have been proposed to explain their effect on neurons and their role in neurodegeneration and dementia.

Disturbed neuronal calcium ion homeostasis could play a subordinate but also an essential role in the neurodegeneration that causes AD (for reviews, see Refs. 2 and 25–27). Indications of the nature of the alterations, caused either by PS1 itself or by APP cleavage products, was analyzed by us and others in relevant paradigms, *i.e.* neurons and brain sections derived from transgenic mice (5, 6, 7, 21). We have now addressed these questions directly by comparatively analyzing neurons derived from six different transgenic mouse strains expressing either mutant PS1 or mutant APP on APP- or PS1-deficient backgrounds (Table I).

First, we present experimental evidence that APP is not directly involved in CCE or ER-based calcium ion storage, a finding with major implications for the physiological function of APP. In contradiction to results obtained in fibroblasts derived from APP−/− mice (27), we did not observe any alterations in cultured hippocampal neurons from APP−/− mice in response to thapsigargin or bradykinin. By analyzing CCE in double transgenic mice that express the mutant PS1 on an APP−/− background, we observed that the attenuation of CCE by mutant PS1 is not dependent on the expression of APP (Table I). The attenuation of CCE by mutant PS1 must then be due to a direct interference with the mechanism of CCE that is independent of APP and APP-cleavage and hence independent of the γ-secretase activity of PS1. The conclusion that a normal physiological function of PS1 is to be sought in the regulation of CCE and the refilling of the ER-calcium stores could be structurally explained if PS1 participates in the formation of a channel within the plasma and/or ER-membrane that mediates calcium ion movements. This proposition finds strong support in the fact that PS1 is present at the cell surface (28), which was most recently and convincingly confirmed and functionally extended to other partners, *i.e.* nicastrin (29). The implication of a close and intricate structural relationship of the plasma and ER membranes in neurons to the function of neuronal signaling and calcium homeostasis as well as in terms of the possible contribution to the molecular basis of memory and neurodegeneration was proposed and discussed extensively (Refs. 8, 25, 30, and references therein). There is clearly a need for the experimental demonstration that PS1 takes part in a novel structural entity linking the plasma and the ER membranes to establish the molecular identity of the proposed structures now diversely known as "plasmerosomes" or "store-operated channels" (Refs. 8, 25, 30, and references therein). Evidently in this respect as in many others, neurons are structurally and functionally much more complex and differently organized than fibroblasts or other simple cell types. This may explain the divergent results we obtained here in APP−/− neurons relative to APP−/− fibroblasts (27).

In contrast to the direct attenuation of CCE by mutant PS1, we present robust evidence that the increase of ER-based calcium stores by mutant PS1 indeed requires the expression of APP. In the double transgenic mice that express mutant PS1 on the APP−/− background, the deficit in ER calcium signaling

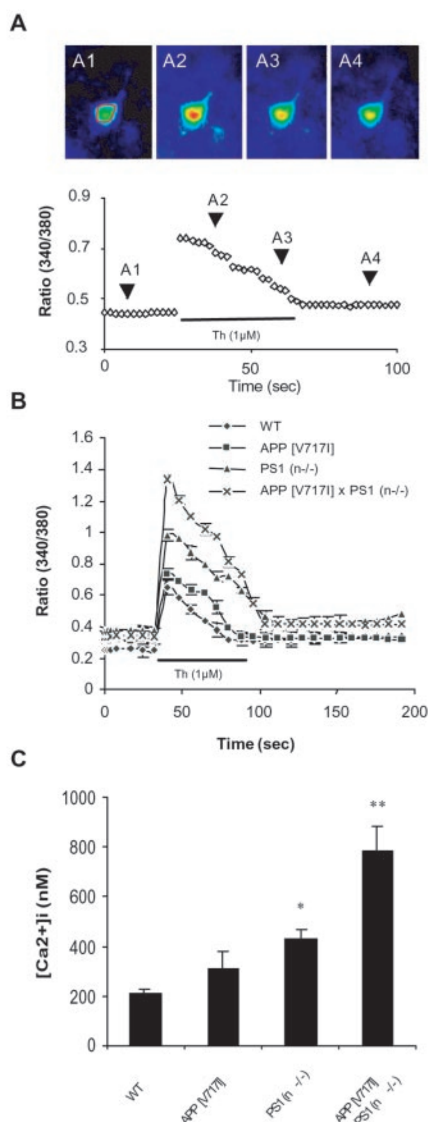


FIG. 4. **Increased ER-calcium stores in hippocampal sections from PS1(n−/−) and from APP[V717] × PS1(n−/−) mice.** *A*, digital micrographs of a hippocampal pyramidal cell in an acute slice preparation loaded with fura-2 by a patch pipette as described under the "Experimental Procedures." The images are at the different time points indicated in the 340/380 nm fluorescence ratio tracing, calculated over the area indicated in *section A1* of *panel A*. *B* and *C*, response to thapsigargin (*Th*) of $[Ca^{2+}]_i$ in APP[V717I] neurons is not significantly different from that in wild-type neurons, whereas the increase in PS1-deficient neurons (PS1(n−/−) (*, $p < 0.01$) is strongly accentuated by the expression of APP[V717I] (**, $p < 0.001$). Thapsigargin was applied by superfusion (2 ml $min^{-1}$) as indicated by the *bar* (*panel B*). Data points in *panel B* are mean fluorescence ratios ± S.E. ($n$ = 50–55 cells), whereas in *panel C* the peak values of $[Ca^{2+}]_i$ are given (*, $p < 0,05$; **, $p < 0,001$).

was restored to normal values. This result evidently excludes the possibility that APP-dependent alterations in ER calcium signaling are responsible for the depression of CCE by mutant PS1, as proposed (5). The argument that higher levels of ER calcium ions in PS1 mutant cells would impair CCE by preventing the agonists from depleting the intracellular calcium stores beyond a threshold required to activate CCE (5) is contradicted by our finding that CCE is still attenuated by mutant PS1 in the APP−/− double transgenic mice, whereas the filling of ER-calcium stores is not altered in the absence of APP (Table I).

It is clear that the thapsigargin-sensitive calcium ion movements in mutant PS1 neurons are dependent on the presence of APP, leading to the conclusion that this involves the γ-secretase activity of PS1 and therefore the proteolytical fragments of APP, *i.e.* amyloid peptides and their obligatory precursors, the C99 fragments. The analysis of PS1-deficient neurons yielded, to our surprise, similar alterations in ER calcium storage as observed in mutant PS1 neurons. Because amyloid peptide production is very low in PS1−/− neurons (4, 9), we must conclude that the C99 fragments mediated the observed alterations of the ER calcium signaling. The relative changes in the size of the ER-calcium ion stores sensitive to thapsigargin correlate very well with the relative changes in the level of the amyloid and C99 fragments in the brains of the mice analyzed (Table I, *right panel*) (9–12).

The critical role of the C99 fragments is corroborated by the proposed neurotoxicity of these fragments as demonstrated in cell biological models (Refs. 2, 26, and references therein). Most recently, we have obtained the first indications for the same effect *in vivo* in adult mice that lack neuronal PS1 (9). It is not clear, however, how the amyloid and C99 fragments disturb the ER-based calcium ion stores. In transfected cells, the C57 fragment of APP appeared to induce similar alterations in ER calcium stores as the C99 fragment (27). It was proposed that the C57 fragments interact with the nuclear adaptor protein Fe65, which, in turn, interacts with transcription factors (31, 32). This would mean that alterations in ER calcium storage in PS1 transgenic mice are due to transcriptional effects of the C57 intracellular cleavage product of APP, *i.e.* the APP intracellular domain (AICD), in complete analogy to the modes of generation and action of the intracellular cleavage product of Notch (NICD) (31). Although AICD was below detection limits in the brains of the transgenic mice studied here (results not shown), we can evidently not rule them out. Conversely, we observed increased ER calcium ion storage in PS1−/− neurons in which the generation of AICD is strongly inhibited. This contradicts results obtained by others in fibroblasts (27) and primary neurons derived from conventional PS1-knockout mice (6). Possible factors contributing to this discrepancy include the fact that the latter PS1−/− neurons were derived from embryonic tissues from total PS1 knockout mice, whereas we derived primary neuronal cultures from postnatal conditional PS1 knockout mice in which the floxed PS1 gene is inactivated by coexpressed Cre recombinase driven by the *thy1* gene promoter (9). However, here we also analyzed the response to thapsigargin in a different experimental setting, *i.e.* in hippocampal slices derived from adult PS1(n−/−) mice and demonstrated a very similar effect as in the postnatal primary cultured neurons.

The most direct explanation of the similar results we obtained in neurons that either express mutant PS1 or are deficient in PS1 would be that the gain of function of mutant PS1 leads to increased levels of the AICD or C57 fragments, whereas the loss of function of PS1 increases the C99 fragments. But then we must accept that the C99 and the C57 fragments act similarly with respect to calcium homeostasis, which could be at the transcriptional level. This requires additional experimental definition of the mechanisms involved in both types of genetically modified neurons.

The problem of whether and how alterations in neuronal calcium homeostasis relate to loss of synaptic functions and eventually to nerve cell loss in Alzheimer's disease has been speculated upon before (for a review, see Ref. 2). Based on results from experiments performed with fibroblasts (33) and lymphocytes (34) from AD patients carrying a mutant PS1 gene, it was proposed that neurons in these patients might suffer from alterations in ER calcium ion homeostasis well before the patients develop the mental disorder. Alterations in intracellular free calcium concentration have also been impli-

cated in other neurodegenerative diseases, *i.e.* prion disease (35) and amyotrophic lateral sclerosis (36) as well as normal aging (37).

In conclusion, we analyzed neurons derived from the six most relevant single and combined transgenic and gene-deficient mouse strains to demonstrate that altered neuronal calcium dynamics caused by mutant PS1 are dual in nature. First, the hypothesis that mutant PS1 attenuates CCE directly, independent of APP as proposed (6), is now proven by the data presented here. Secondly, as opposed to CCE, the increase of ER-calcium stores by mutant PS1 is found to be dependent on APP and is correlated closely with the combination of changes in the levels of the amyloid peptides and the C99 fragments of APP. How these fragments alter ER-calcium stores is a matter of debate. The C99 fragments might act either directly and similarly to the amyloid peptides, or they might be further degraded and act like the APP intracellular domain AICD (27). Our observations do not lead to a complete understanding of the normal physiological roles of APP and PS1, which are clearly much more complex and diverse than anticipated from the pathological consequences of their malfunction. Conceivably, the pathogenic consequences of perturbed calcium homeostasis also offer possibilities for therapeutic approaches. Conversely, the possible benefits of using $\gamma$-secretase inhibitors for the treatment of AD are seriously challenged by the current and previous results, as these inhibitors will disturb neuronal calcium homeostasis by increasing the levels of the N-terminal fragments of APP (9).

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