

Mutant Presenilins Disturb Neuronal Calcium Homeostasis in the Brain of Transgenic Mice, Decreasing the Threshold for Excitotoxicity and Facilitating Long-term Potentiation*

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Iika Schneider^{‡§}, Delphine Reversé^{§¶}, Ilse Dewachter^{§||**}, Laurence Ris^{¶‡‡},
Nathalie Caluwaerts[¶], Cuno Kuiperi[¶], Martine Gilis[¶], Hugo Geerts^{§§}, Hans Kretzschmar[‡],
Emile Godaux[¶], Dieder Moechars^{§§}, Fred Van Leuven^{¶¶}, and Jochen Herms[‡]

From the [‡]Department of Neuropathology, Ludwig-Maximilians-Universität, 81377 Munich, Germany, [¶]Laboratory of Neuroscience, University of Mons-Hainaut, 7000 Mons, Belgium, ^{||}Experimental Genetics Group, Katholieke Universiteit Leuven, 3000 Leuven, Belgium, and the ^{§§}Janssen Research Foundation, 2340 Beerse, Belgium

Mutant human presenilin-1 (PS1) causes an Alzheimer's-related phenotype in the brain of transgenic mice in combination with mutant human amyloid precursor protein by means of increased production of amyloid peptides (Dewachter, I., Van Dorpe, J., Smeijers, L., Gilis, M., Kuiperi, C., Laenen, I., Caluwaerts, N., Moechars, D., Checler, F., Vanderstichele, H. & Van Leuven, F. (2000) *J. Neurosci.* 20, 6452–6458) that aggravate plaques and cerebrovascular amyloid (Van Dorpe, J., Smeijers, L., Dewachter, I., Nuyens, D., Spittaels, K., van den Haute, C., Mercken, M., Moechars, D., Laenen, I., Kuiperi, C., Bruynseels, K., Tesseur, I., Loos, R., Vanderstichele, H., Checler, F., Sciot, R. & Van Leuven, F. (2000) *J. Am. Pathol.* 157, 1283–1298). This gain of function of mutant PS1 is approached here in three paradigms that relate to glutamate neurotransmission. Mutant but not wild-type human PS1 (i) lowered the excitotoxic threshold for kainic acid *in vivo*, (ii) facilitated hippocampal long-term potentiation in brain slices, and (iii) increased glutamate-induced intracellular calcium levels in isolated neurons. Prominent higher calcium responses were triggered by thapsigargin and bradykinin, indicating that mutant PS modulates the dynamic release and storage of calcium ions in the endoplasmic reticulum. In reaction to glutamate, overfilled Ca^{2+} stores resulted in higher than normal cytosolic Ca^{2+} levels, explaining the facilitated long-term potentiation and enhanced excitotoxicity. The lowered excitotoxic threshold for kainic acid was also observed in mice transgenic for mutant human PS2[N141I] and was prevented by dantrolene, an inhibitor of Ca^{2+} release from the endoplasmic reticulum.

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§ these authors contributed equally to this work.

** Post-doctoral fellow of the KU Leuven research fund.

‡‡ Research assistant of the Belgian National Fund for Scientific Research.

¶¶ To whom correspondence should be addressed: Experimental Genetics Group, Center for Human Genetics, Campus Gasthuisberg O&N 06, B-3000 Leuven, Belgium. Tel.: +32-16-3458-62; Fax: +32-16-3458 71; E-mail: fredvl@med.kuleuven.ac.be.

Similar post-mortem diagnostic features in all Alzheimer's disease (AD)¹ patients, sporadic or familial, suggest common pathogenic mechanisms. Dominant early onset familial AD (EOFAD) is mainly caused by mutant presenilin1 (PS1) (3), which like mutant amyloid precursor protein (APP) increase the production of amyloid peptides (A β), especially A β 42 (4). Presenilins (PS) contain 6 to 8 transmembrane domains and are predominantly confined to the endoplasmic reticulum (ER). Similar to *Caenorhabditis elegans sel-12* and *spe-4*, PS might function in signal transmission and intracellular transport (5, 6). More recently PS1 was claimed to be γ -secretase (7), the elusive proteinase responsible for cleaving A β from APP (8).

Amyloid deposits and neurofibrillary tangles are the obligatory diagnostic lesions in all AD patients. This duality is not explained by an increased production of A β , the proposed primary defect in EOFAD, although perturbation of intracellular fluxes of calcium ions could be the main problem in sporadic AD (9). Whether this causes or follows from increased A β , by affecting APP processing and/or clearance, is disputed (10–14). Others took failing calcium homeostasis even further to include apoE4 (15, 16), while even more provocative is the "reversed amyloid hypothesis" in which A β and neurofibrillary tangles are "executors" following disrupted calcium homeostasis (17).

The profound repercussions for diagnosis and therapy of the exact mechanisms in AD are evident. To study the pathological mechanism *in vivo*, we generated transgenic mice that overexpress human mutant PS. Very unlike humans, EOFAD mutant PS1 or PS2 transgenic mice show no strong phenotype or pathological defects. Only in combination with human APP do mutant PS increase the production of A β , resulting in more amyloid plaques earlier in life (4, 18, 19) and aggravating cerebrovascular angiopathy (2) by a mechanism different from aging (1). Recently, enhanced long-term potentiation (LTP) or facilitated synaptic transmission was observed and related to altered calcium homeostasis in brain slices of mutant PS1 transgenic mice (12, 20–24). This finding enforced our ongoing analysis of PS1 and PS2 transgenic mice, focusing on glutamate-mediated neurotransmission, thought to be compromised in AD patients (25).

¹ The abbreviations used are: AD, Alzheimer's disease; PS, presenilin(s); EOFAD, dominant early onset familial Alzheimer's disease; APP, amyloid precursor protein; ER, endoplasmic reticulum; A β , amyloid peptide; LTP, long-term potentiation; KA, kainic acid; fEPSP, excitatory postsynaptic potentials; PIPES, 1,4-piperazinediethanesulfonic acid; DMEM, Dulbecco's minimal essential medium; NMDA, *N*-methyl-D-aspartic acid; IP₃, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor.

We studied three paradigms that yield converging and convincing evidence for disrupted neuronal calcium ion homeostasis by mutant PS. The seizure threshold for the excitotoxin kainic acid (KA) was lower, whereas neuronal damage in the hippocampus was prevented by dantrolene, an inhibitor of calcium ion release from the ER. Standard hippocampal LTP was normal in PS1 transgenic mice as opposed to APP transgenic mice (26) but was facilitated when evoked by weak tetanic stimulation. Finally, dynamic changes in intracellular $[Ca^{2+}]_i$ in cultured neurons were disturbed by mutant PS1 by a mechanism that involved the filling and release of Ca^{2+} from stores in the ER, the cellular organelle where PS reside predominantly.

EXPERIMENTAL PROCEDURES

Transgenic Mice—PS1 cDNA, wild-type or A246E mutant (5), and wild-type or mutant N141I PS2 cDNA (27) were spliced in the mouse *thy-1* gene (28) to generate transgenic mice by our standard methods (1, 2, 26, 28). Founders and offspring were genotyped by polymerase chain reaction and Southern blotting of tail-tip DNA. Transgenic strains were established and maintained in the FVB/N background and back-crossed to obtain mice homozygous for either wild-type or mutant human PS1 or PS2.

Analysis of Expression—Northern blots on total brain mRNA were scanned and quantified (1, 26, 28). Western blots of mouse brain extracts, *i.e.* in 5 mM Tris, 250 mM sucrose, 1 mM EGTA (pH 7.4) containing a mixture of proteinase inhibitors. Homogenates were cleared ($12,000 \times g$, 10 min, 4 °C) before membranes were pelleted by high speed centrifugation ($100,000 \times g$, 30 min, 4 °C). Membrane proteins were denatured and reduced, analyzed on 4–20% Tris-glycine polyacrylamide gels (Novex, San Diego, CA), and transferred to nitrocellulose filters. N- and C-terminal fragments of PS1 and PS2 were detected with polyclonal antibodies B14/5, B17/2, and B24/2 as described (1, 29).

Reactivity to Kainic Acid—The doses of KA injected intraperitoneally were established in preliminary experiments to elicit seizures reproducibly with only moderate neuronal damage. Mice were observed for 2 h postinjection, and the frequency and intensity of seizures were rated in seven stages: lethargy, rigid posture, head bobbing or circling, clonic seizure, rearing alone or with falling, tonic-clonic seizures, and death. Pretreatment with dantrolene (10 mg/kg intraperitoneal) was for 30 min prior to injection of KA (30). Histology for hippocampal damage was done 24 h after KA injection on perfused and fixed brains (4% paraformaldehyde) dehydrated and embedded in paraffin. Sections (7 μ m) were stained with hematoxylin-eosin and cresyl violet for hippocampal damage and scored on a scale from 1 to 5 (31): 1 = minor damage to some pyramidal cells in CA1 or CA3; 2 = mild damage to a small number of pyramidal cells in CA1 or CA3; 3 = moderate damage to a larger area in one hippocampal region (CA1 or CA3); 4, severe damage to two hippocampal regions; 5 = extreme damage or substantial neuronal death in more than two regions, *i.e.* CA1, CA3, CA4, or dentate gyrus. Sections were scored blind and independently by two investigators.

Electrophysiology—Transverse vibratome sections (400 μ m) were at all times bathed in artificial cerebrospinal fluid, *i.e.* 124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.24 mM KH_2PO_4 , 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM glucose, saturated with 95% O_2 and 5% CO_2 . Electrophysiological recordings, done at least 3 h after dissection to allow recovery, were made with bipolar tungsten microelectrodes to stimulate Schaeffer's collaterals. Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in CA1 with low resistance (2 megohm) glass microelectrodes filled with 2 M NaCl. Test stimuli were 0.1-ms constant voltage pulses delivered every 30 s at an intensity sufficient to evoke an ~33% maximal response. LTP was induced by high frequency stimulation either 2 trains of 1-s pulses of 100 Hz separated by 20 s with each pulse 0.2 ms (strong stimulation), or a single train of 0.4 s, 100 Hz, with each pulse 0.1 ms (weak stimulation). The slope of fEPSP (mV/ms) was averaged from four consecutive responses. Paired-pulse facilitation was measured by the relative ratio of the slope of the second to the first fEPSP.

Acute Dissociation of Hippocampal Neurons from Adult and Newborn Mice—Neurons from adult mouse hippocampi (32) were dissociated from sections (200 μ m) incubated at 32 °C in oxygen-saturated buffer (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM PIPES, 25 mM glucose (pH 7.0) containing 0.8 mg/ml trypsin) for 90 min. Slices were rinsed, triturated in Dulbecco's minimal essential medium (DMEM) with fire-polished glass pipettes, and plated on polylysine-coated coverslips. Neurons were allowed to attach, and the medium was replaced by HEPES-buffered saline used for dye loading and recording.

Primary hippocampal neurons from newborn mice were plated on astrocyte monolayers treated with the antimetabolic agents 5-fluoro-2-deoxyuridine (8.1 mM) and uridine (20.4 mM). Astrocyte feeder layers were prepared from the spinal cords of newborn mice by mechanical and enzymatic dissociation with trypsin. Cells were suspended in DMEM with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cells were plated (3×10^3 cells/well) in 4-well cluster plates on coverslips coated with polylysine and collagen.

Hippocampi from newborn mice were dissociated with papain (25 units/ml) activated with cysteine (2 mM) in DMEM for 60 min at 37 °C before transfer to DMEM with fetal calf serum (5%), N2 supplement, and antibiotics. Cells were triturated and plated on astrocyte feeder layers. After attachment (3–6 h after plating), the medium was changed to neurobasal medium with B27 supplement (Life Technologies, Inc.). Experiments were performed on neurons cultured for 19–21 days in Locke's buffer, *i.e.* 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, 5 mM HEPES (pH 7.2).

Microfluorometric Calcium Measurements—Cytoplasmic calcium was measured by fluorescence ratio imaging with acetoxymethyl-fura 2 (fura-2/AM). For dye loading, cells on coverslips were incubated in Locke's buffer with Fura-2/AM (5 μ M) for 30 min at 37 °C, washed, and imaged under an upright microscope. Average intracellular calcium ion concentration ($[Ca^{2+}]_i$) in individual neurons was calculated from fluorescence using a digital imaging system (Till Photonics, Munich, Germany). Measurements were acquired at 1–3-s intervals at dual excitation wavelengths, and digital fluorescence images were constructed. $[Ca^{2+}]_i$ was determined for each pixel in the frame with fluorescence intensities over a given threshold and using the relation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_0/F_s)$ (33).

RESULTS

Transgenic Mice Expressing Wild-type and Mutant Presenilins—Transgenic mice that express either human wild-type PS1 or PS2 or EOFAD mutants PS1[A246E] or PS2[N141I] were generated using the mouse *thy-1* gene promoter (28) to assure expression in neurons only (1, 2, 26). For each construct, 2–6 independent founders were generated, and the selection of strains was based on human transgene mRNA and protein levels in brain (results not shown). Human PS transgenic proteins were present at levels similar to endogenous murine PS1 in wild-type mouse brain, indicating that saturating replacement was effective (see Refs. 1 and 2 for references and discussion). Western blotting demonstrated proteolytic processing of human PS1 transgenes into N- and C-terminal fragments (1, 2) (results not shown). For current experiments we used 2 transgenic lines expressing PS1[A246E], *i.e.* lines 2 and 4 compared with 2 PS1[wt] transgenic strains 7 and 8. APP metabolism was disturbed in the brain of double transgenic mice that coexpress mutant PS1[A246E] and human APP/London (1, 2). Histologically, the brains of all PS transgenic mice, including PS1[A246E] and PS2[N141I] mice up to 2 years old, were normal by standard hematoxylin-eosin, silver, and thioflavin-S staining (1, 2) (results not shown). Learning and spatial memory of PS1[A246E] transgenic mice were unaffected in the water maze test, in which neither the escape latency nor escape pathway was different from PS1[wt] transgenic mice at 3 and 9 months of age (1, 2) (results not shown).

Excitotoxicity in Response to Kainic Acid—KA elicited significant higher seizure activity for the same dose in 3 independent strains of mutant transgenic mice, *i.e.* 2 strains of PS1[A246E] and the PS2[N141I] strain. Wild-type human PS1 or PS2 transgenic mice reacted in a manner similar to nontransgenic mice (Table I). Neuronal damage 24 h after KA was rated on a scale of 1 to 5 (specified under "Experimental Procedures"). In the hippocampus of nontransgenic mice, confirmed as the area most vulnerable to KA (31), the average neuron death varied from minor to mild for doses of 12 and 16 mg KA/kg, respectively (Table II). Neuronal damage was confined largely to single or small clusters of pyramidal cells in CA3, known to be most susceptible to KA (31, 34). Damage was always absent from the CA2 and dentate gyrus in all mice, confirming that

TABLE I
Seizures

Dose	Nontransgenic	PS1 (wild type)		PS1[A246E]		PS2 (wild type)	PS2[N141I]
		Strain 7	Strain 8	Strain 2	Strain 4		
$\mu\text{g/g}$							
12	31.5 (3.8) <i>n</i> = 16	30.1 (4.5) <i>n</i> = 8, <i>p</i> = 0.83	33.1 (4.1) <i>n</i> = 8, <i>p</i> = 0.79	50.9 (4.2) <i>n</i> = 7, <i>p</i> = 0.007	47.8 (6.0) <i>n</i> = 9, <i>p</i> = 0.025	32.5 (6.4) <i>n</i> = 6, <i>p</i> = 0.83	57.6 (3.9) <i>n</i> = 9, <i>p</i> = 0.0001
16	52.4 (5.3) <i>n</i> = 14	56.7 (7.2) <i>n</i> = 14, <i>p</i> = 0.64	60.3 (7.2) <i>n</i> = 11, <i>p</i> = 0.38	75.5 (8.3) <i>n</i> = 11, <i>p</i> = 0.02	71.1 (6.9) <i>n</i> = 10, <i>p</i> = 0.04	58.8 (8.6) <i>n</i> = 9, <i>p</i> = 0.51	81.7 (10.9) <i>n</i> = 6, <i>p</i> = 0.014
20	83.1 (3.9) <i>n</i> = 13						

TABLE II
Neuronal damage

Dose	Nontransgenic	PS1 (wild type)		PS1[A246E]		PS2 (wild type)	PS2[N141I]
		Strain 7	Strain 8	Strain 2	Strain 4		
$\mu\text{g/g}$							
12	1.16 (0.22) <i>n</i> = 19	1.38 (0.30) <i>n</i> = 16, <i>p</i> = 0.56	1.67 (0.36) <i>n</i> = 15, <i>p</i> = 0.22	2.17 (0.51) <i>n</i> = 12, <i>p</i> = 0.046	2.18 (0.33) <i>n</i> = 17, <i>p</i> = 0.014	1.00 (0.45) <i>n</i> = 10, <i>p</i> = 0.72	2.59 (0.39) <i>n</i> = 17, <i>p</i> = 0.0025
16	1.68 (0.32) <i>n</i> = 25	1.70 (0.35) <i>n</i> = 20, <i>p</i> = 0.97	1.60 (0.31) <i>n</i> = 20, <i>p</i> = 0.86	3.33 (0.53) <i>n</i> = 12, <i>p</i> = 0.008	3.61 (0.24) <i>n</i> = 18, <i>p</i> = 0.00006	2.11 (0.48) <i>n</i> = 18, <i>p</i> = 0.44	4.45 (0.21) <i>n</i> = 11, <i>p</i> = 0.000001
20	3.05 (0.38) <i>n</i> = 20						

TABLE III
Dantrolene

	Nontransgenic		PS2[N141I]	
	Dantrolene		Dantrolene	
Seizure score	52.4 (5.3) <i>n</i> = 14	49 (4.6) <i>n</i> = 10, <i>p</i> = 0.6	81.7 (10.9) <i>n</i> = 6, <i>p</i> = 0.014	71.6 (6.8) <i>n</i> = 10, <i>p</i> = 0.4
Neuronal damage	1.68 (0.32) <i>n</i> = 25	1.50 (0.40) <i>n</i> = 20, <i>p</i> = 0.7 ^a	4.45 (0.21) <i>n</i> = 11, <i>p</i> = 0.000001 ^b	1.60 (0.36) <i>n</i> = 20, <i>p</i> = 0.000005 ^c

^a *p* value of nontransgenic mice versus nontransgenic mice treated with dantrolene.

^b *p* value of PS2[N141I] transgenic mice versus nontransgenic mice.

^c *p* value of PS2[N141I] transgenic mice versus PS2[N141I] transgenic mice treated with dantrolene.

these structures are unaffected by KA (35, 36).

Considerably more neuronal damage was evident in mutant PS1 and PS2 transgenic mice. In two different PS1[A246E] strains and in PS2[N141I] mice, average neuronal damage at each dose of KA was significantly higher than in nontransgenic or wild-type PS1 or PS2 transgenic mice (Table II; results not shown).

Dantrolene (30) administered prior to KA in PS2[N141I] transgenic mice significantly reduced or even completely prevented neuronal damage from KA, with only a minimal lowering of seizure activity (Table III). In a smaller scale experiment, mutant PS1 transgenic mice were also protected against KA by dantrolene (results not shown).

Hippocampal LTP—Hippocampal LTP was measured at synapses between Schaeffer's collaterals and CA1 pyramidal neurons (26, 37, 38). LTP evoked by strong tetanic stimulation was not different between PS1 transgenic mice (Fig. 1A). In contrast, weaker stimulation, *i.e.* 1 train of 0.4 s at 100 Hz with 0.1-ms pulses, induced stronger LTP in the hippocampus of PS1[A246E] transgenic as opposed to PS1[wt] transgenic mice or nontransgenic mice. After 2 h the fEPSP slope in PS1[A246E] transgenic mice was $169.2 \pm 30.3\%$ as opposed to $119.9 \pm 18.2\%$ in PS1[wt] transgenic mice ($p < 0.05$) (Fig. 1B).

Paired-pulse facilitation, a reflection of the amount of neuromediator ejected because of the residual increase in $[\text{Ca}^{2+}]_i$ in presynaptic terminals after the first pulse, was measured using interpulse intervals ranging from 25 to 100 ms. Independent of the interval, paired-pulse facilitation was similar in all mice. At 50-ms interpulse intervals, the ratio (second to first stimulus) of slopes of fEPSP was $168.7 \pm 36.1\%$ in nontransgenic mice, $163.7 \pm 22.9\%$ in PS1[A246E] transgenic mice, and

$165.5 \pm 20.2\%$ in PS1[wt] transgenic mice.

$[\text{Ca}^{2+}]_i$ in Acutely Dissociated Neurons of Adult Mice—No significant differences were observed in basal $[\text{Ca}^{2+}]_i$ in the neurons of adult and aged PS1[wt] and PS1[A246E] mice. Potassium ions triggered a rapid increase in $[\text{Ca}^{2+}]_i$ in the soma of adult PS1[wt] and PS1[A246E] neurons. Peak values (1–3 s) were significantly higher in PS1[A246E] neurons (Fig. 2), whereas in neurons derived acutely from old transgenic mice even higher values were measured. The normalization of medium potassium ion levels led to a progressive decline of $[\text{Ca}^{2+}]_i$ to initial resting levels (Fig. 2). Glutamate (50 μM) increased peak $[\text{Ca}^{2+}]_i$ significantly more in PS1[A246E] relative to PS1[wt] neurons (Fig. 3). Again, in neurons from old PS1[A246E] transgenic mice, the peak $[\text{Ca}^{2+}]_i$ induced by glutamate was significantly higher than in young PS1[A246E] mice (Fig. 2). PS1[A246E] hippocampal neurons, in parallel with PS1[wt] neurons exposed to increasing concentrations of glutamate, were much more sensitive to glutamate-triggered excitotoxicity (Fig. 3). This finding corroborated our own *in vivo* observations (see above) and reported results on hippocampal neurons from unrelated PS1[M146V] transgenic mice (12).

$[\text{Ca}^{2+}]_i$ in Cultured Neurons—In cultured hippocampal neurons from newborn mice, basal $[\text{Ca}^{2+}]_i$ was similar independent of the mouse genotype (Fig. 4). Potassium ions caused a rapid $[\text{Ca}^{2+}]_i$ increase in all cultures with a similar time course; *i.e.* similar initial peak after depolarization followed by a rapid decline to a plateau that was somewhat lower in PS1[A246E] neurons (Fig. 4). The wash-out of potassium ions progressively restored $[\text{Ca}^{2+}]_i$ to initial resting levels in all cultures of nontransgenic, PS1[wt] and PS1[A246E] neurons (Fig. 4).

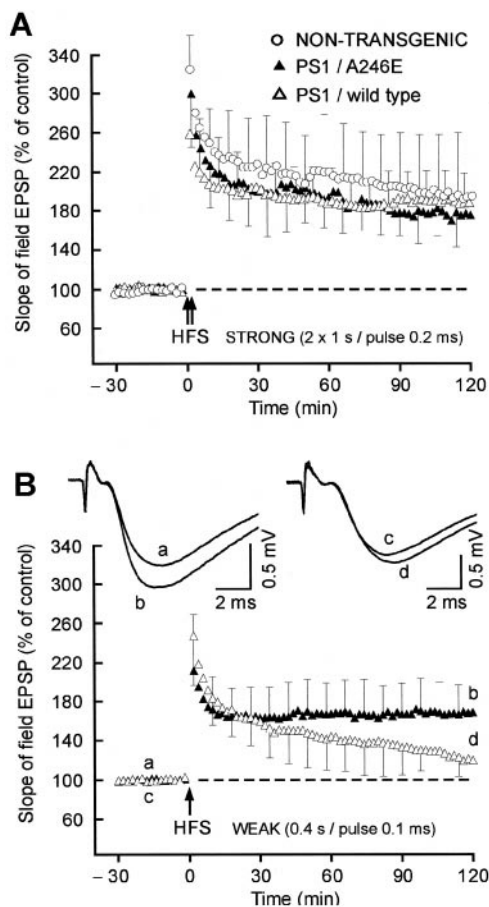


FIG. 1. Hippocampal LTP induced by strong and weak stimulation. *A*, relative changes in fEPSP slopes as a function of time after strong high frequency stimulation, *i.e.* two trains of 1 s with pulses of 0.2 ms at 100 Hz. All points are the mean \pm S.E. of 6 experiments on sections from 6 distinct mice with the indicated genotype. *B*, relative changes in fEPSP slopes as a function of time after weak HFS stimulation, *i.e.* trains of 0.4 s at 100 Hz with 0.1-ms pulses. *Inset*, examples of typical fEPSP recorded 2 h after HFS (*traces b* and *d*) superimposed on control fEPSP (*traces a* and *c*) for PS1[A246E] transgenic mice (*left*) and PS1[wt] transgenic mice (*right*).

Again, glutamate induced a rise in $[Ca^{2+}]_i$ that was significantly higher in neurons from PS1[A246E] mice (Fig. 4) than in acutely dissociated adult neurons (Fig. 2). The addition of NMDA (70 μ M with 10 μ M glycine) resulted in comparable responses in PS1[wt] and PS1[A246E] hippocampal neurons (Fig. 4C) indicating that NMDA receptors did not contribute differentially.

Bradykinin (1 μ M) increased $[Ca^{2+}]_i$ significantly more in PS1[A246E] neurons than in nontransgenic and PS1[wt] transgenic neurons (Fig. 4D). This extends the findings in fibroblasts from PS1[M146V] transgenic mice (24) to the most relevant cell type and demonstrates that these responses are mediated by metabotropic glutamate receptors. IP₃ receptor protein levels were not different in the brains of wild-type and transgenic mice on Western blots (data not shown).

The depletion of the ER stores with thapsigargin (1 μ M) significantly increased $[Ca^{2+}]_i$ in PS1[A246E] hippocampal neurons relative to PS1[wt] neurons (Fig. 4E). Collectively, the data indicate that the homeostasis of intracellular Ca^{2+} was severely disturbed by mutant presenilins by a mechanism(s) that involves the dynamics of ER calcium stores.

DISCUSSION

Overexpression of EOFAD mutants PS1[A246E] or PS2[N141I] in the brain of transgenic mice resulted in no major pathological

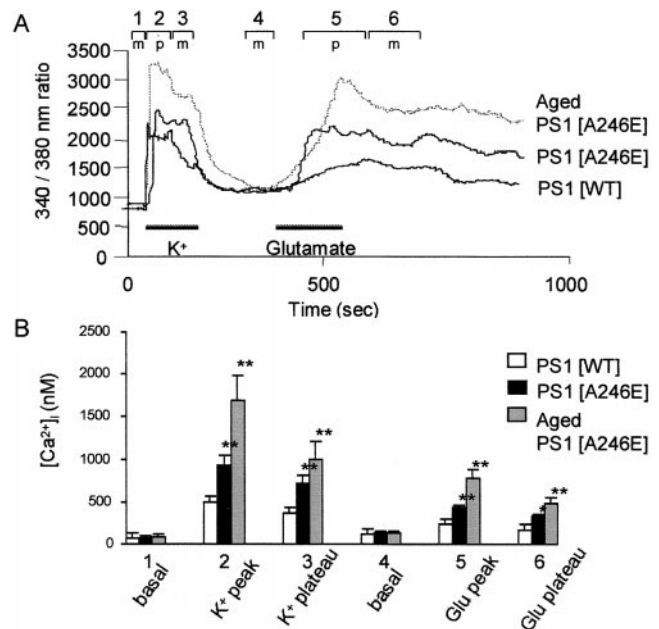


FIG. 2. Effects of potassium depolarization and glutamate on $[Ca^{2+}]_i$ in acutely dissociated neurons. *A*, typical changes in $[Ca^{2+}]_i$ in individual cells of acutely dissociated hippocampal neurons from adult PS1[wt] and PS1[A246E] mice (4–6 month) and old PS1[A246E] mice (14–16 month). Cells were treated subsequently by 50 mM K^+ and 50 μ M glutamate (indicated by lower bars). *B*, values of the mean (*m*) and peak (*p*) values from 10–15 cells analyzed in four individual mice obtained as indicated in panel *A* (top). Statistical significance: *, $p < 0.01$; **, $p < 0.001$.

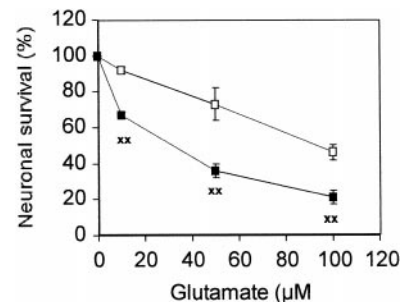


FIG. 3. Effect of glutamate concentration on neuronal survival. Hippocampal neurons from PS1[wt] (*open squares*) and PS1[A246E] transgenic mice (*closed squares*) exposed for 24 h to glutamate. Survival was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values are the mean \pm S.E. of 3 cultures. Statistical significance: * $p < 0.01$, ** $p < 0.001$.

manifestations, even in old mice, as observed by us and others (1, 2, 12, 39). This is remarkably different from humans, because mutant PS1 causes the most aggressive cases of EOFAD (40). On the other hand, in combination with human APP, PS1[A246E] was extremely pathogenic, *i.e.* it increased amyloid in brain parenchyma and cerebral blood vessels (Refs. 1 and 2 and references therein). The proposed relationship of PS1 to γ -secretase (7, 8) should not distract from the fact that the exact gain-of-function of mutant presenilins *in vivo* is unknown. Therefore, PS1[A246E] and PS2[N141I] transgenic mice were analyzed for (i) reactivity to kainic acid, (ii) hippocampal LTP, and (iii) regulation of $[Ca^{2+}]_i$. In all three paradigms, glutamate-mediated neurotransmission is implicated as in the pathophysiology of AD by the concept of excitotoxicity (25).

Mice expressing mutant PS1[A246E] or PS2[N141I] were more sensitive to kainic acid as evident from the acute seizure intensity and delayed neuronal damage. The neurotoxicity of KA obligatory involves glutamate receptors and results in cellular Ca^{2+} overload, confirmed by direct measurements here

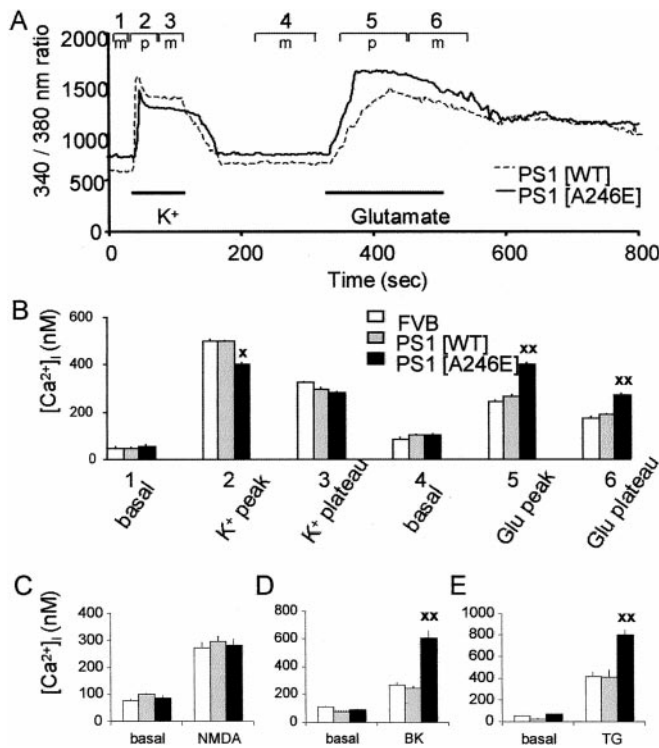


FIG. 4. Effect of potassium depolarization and glutamate on $[Ca^{2+}]_i$ in primary hippocampal neurons. *A*, typical changes in $[Ca^{2+}]_i$ in individual cell somata of primary cultured hippocampal neurons from newborn nontransgenic mice (FVB) and from PS1[wt] and PS1[A246E] transgenic mice. See Fig. 3 for experimental details. *B*, mean (*m*) and peak (*p*) values from cells ($n = 20$ – 30) analyzed at the time intervals indicated in panel *A*. Statistical significance: *, $p < 0.01$; **, $p < 0.001$. *C*, $[Ca^{2+}]_i$ basal and following the addition of NMDA ($70 \mu M$ NMDA with $10 \mu M$ glycine) in at least 20 cells from 4 cultures (mean \pm S.E.). *D* and *E*, peak $[Ca^{2+}]_i$ in response to bradykinin (BK, $1 \mu M$) (*D*) and thapsigargin (TG, $1 \mu M$) (*E*) in hippocampal neurons of nontransgenic (FVB) and PS1[wt] and PS1[A246E] transgenic mice. The mean \pm S.E. of the rise in $[Ca^{2+}]_i$ in at least 20 cells in 4 independent cultures is shown. Statistical significance: **, $p < 0.001$.

and in mice with a different PS1 mutant (12). Dantrolene, an effective inhibitor of calcium release from the ER, effectively protected mutant PS transgenic mice from excitotoxicity. This finding supports experimentally the notion that mutant PS contribute to neurotoxicity by disturbing calcium ion homeostasis in and from the ER.

This conclusion joined seamlessly with our findings of facilitated induction of LTP and with related aspects of disturbed calcium homeostasis, which were published recently during the course of this work (12, 20–24). Classic LTP was not different in PS transgenic mice when induced by a strong stimulus, in contrast to weak stimulation that elicited LTP only in mutant PS1 mice. Clearly, mutant PS1 decreased the threshold for LTP without affecting its maximum amplitude. The induction of LTP in CA1 operates through Ca^{2+} influx via NMDA receptors and L-type voltage-gated calcium channels (37, 41). Release from the ER is debated and largely based on depletion of internal stores by thapsigargin, which blocks LTP induced by weak but not strong tetanization (42–44). The release of Ca^{2+} from the ER is mediated by IP_3 and ryanodine receptors (RyR). Blocking metabotropic glutamate receptors prevents both the generation of IP_3 and the induction of LTP (45). The effect of bradykinin supports an IP_3 -mediated effect of PS mutants, which are anchored in the ER and could directly effect Ca^{2+} release by IP_3 receptors. LTP is triggered evidently by NMDA receptors, but the reaction of $[Ca^{2+}]_i$ to NMDA was unaffected in mutant PS1 neurons. Although RyR expression might be

disturbed by mutant PS1 (46), the potassium ion depolarization results in cultured mutant PS1 neurons argue against involvement of RyR in our experimental system.

Therefore, the detailed mechanism of $[Ca^{2+}]_i$ increase following glutamate in neurons of PS1[A246E] mice remains to be defined. The significant response of PS1[A246E] neurons (present work), of fibroblasts from PS1[M146V] mice (24), and of PS1 mutant PC12 cells to thapsigargin (47) all suggest an increased pool of Ca^{2+} ions available for release. Although this points to the ER and is most consistent with the localization of PS in the ER, the complex cellular responses to calcium influx make it as yet impossible to identify the primary effect of mutant PS on the dynamic regulation of $[Ca^{2+}]_i$, referring also to capacitive calcium entry (48) or RyR expression (46).

Finally, and most intimately related to AD pathology, we must address the apparent contradiction of mutant PS affecting calcium homeostasis and PS in or as γ -secretase activity (7, 8). It is conceivable that all of the effects on calcium homeostasis of mutant PS are indirect and are mediated by increased cellular levels of amyloid peptides. None of the available systems allows one to accept or dismiss this possibility, whereas on the contrary, very recent evidence strongly supports the opposite view (48). To clarify this hypothesis, we have generated additional mouse strains that express mutant PS1 in an APP-deficient background, in addition to mice that express mutant APP[V717I] in a conditional, neuron-specific PS1-deficient background. These multiple transgenic mouse strains are now being characterized to define whether and how mutant PS1 affects neuronal calcium homeostasis in the absence of amyloid peptides. Increased $[Ca^{2+}]_i$ in cultured cells increased $A\beta$ production (11) while also increasing hyperphosphorylation of protein tau (49). This link to both major neuropathological lesions in AD makes the deregulation of intracellular calcium homeostasis a prime candidate for all AD cases, both familial and sporadic.

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