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Modulation of synaptic plasticity and Tau phosphorylation by wild-type and mutant presenilin1

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Abstract

The function of presenilin1 (PS1) in intra-membrane proteolysis is undisputed, as is its role in neurodegeneration in FAD, in contrast to its exact function in normal conditions. In this study, we analyzed synaptic plasticity and its underlying mechanisms biochemically in brain of mice with a neuron-specific deficiency in PS1 (PS1(n-/-)) and compared them to mice that expressed human mutant PS1[A246E] or wild-type PS1. PS1(n-/-) mice displayed a suble impairment in Schaffer collateral hippocampal long-term potentiation (LTP) as opposed to normal LTP in wild-type PS1 mice, and a facilitated LTP in mutant PS1[A246E] mice. This finding correlated with, respectively, increased and reduced NMDA receptor responses in PS1[A246E] mice and PS1(n-/-) mice in hippocampal slices. Postsynaptically, levels of NR1/NR2B NMDA-receptor subunits and activated α -CaMKII were reduced in PS1(n-/-) mice, while increased in PS1[A246E] mice. In addition, PS1(n-/-) mice, displayed reduced paired pulse facilitation, increased synaptic fatigue and lower number of total and docked synaptic vesicles, implying a presynaptic function for wild-type presenilin1, unaffected by the mutation in PS1[A246E] mice. In contrast to the deficiency in PS1, mutant PS1 activated GSK-3 β by decreasing phosphorylation on Ser-9, which correlated with increased phosphorylation of protein tau at Ser-396–Ser-404 (PHF1/AD2 epitope). The synaptic functions of PS1, exerted on presynaptic vesicles and on postsynaptic NMDA-receptor activity, were concluded to be independent of alterations in GSK-3 β activity and phosphorylation of protein tau. © 2006 Elsevier Inc. All rights reserved.

Keywords: Presenilin; FAD mutation; Synaptic plasticity; NMDA receptor; Synaptic vesicles; Presynaptic; Postsynaptic; Tau

1. Introduction

Many questions regarding the functions exerted by presenilins (PS), PS1 and PS2, either directly or indirectly in synaptic plasticity, affecting both structural and functional characteristics, remain to be explored to the fullest. The essential role of PS1 in γ -secretase activity (De Strooper et al., 1998) is undisputed, as the essential active-site component of the γ -secretase complex containing further nicastrin, aph-1 and pen-2 (Iwatsubo, 2004; Kopan and Ilagan, 2004; Steiner, 2004; Thinakaran and Parent, 2004). The direct

involvement of PS1 in very diverse (patho)physiological processes either as γ -secretase with a tantalizing number of substrates, or as mediator of protein trafficking and calcium homeostasis (Herms et al., 2003; Leissring et al., 2000; Ris et al., 2003; Yoo et al., 2000) needs to be further explored. In this respect, we have been offered an experiment of nature that is as interesting as it is tragic: the familial forms of Alzheimer's disease (AD) that are caused by the many mutations in PS1 are the most early and aggressive in onset and course (Hutton and Hardy, 1997; Schellenberg et al., 2000). Many, if not all of the actions of PS1 were indeed brought to light by studies of the role of mutant PS1 in the pathology in EOFAD, followed by studies of PS1 deficient mice and cells. In addition EOFAD linked to PS1 mutations are clinically more aggressive than mutant amyloid precursor protein (APP), while clinical symptoms do not correlate

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well with A β 42 levels (Murayama et al., 1999), raising the hypothesis that mutant PS1 might contribute additionally to the pathology independent of A β 42 (Herms et al., 2003; Koo and Kopan, 2004; Marjaux et al., 2004; Saura et al., 2004).

Concurrent postnatal neuronal deficiency of PS1 and PS2 in mice, results in neurodegenerative changes that are reminiscent for AD (Feng et al., 2004; Saura et al., 2004). The lack of both presenilins in postnatal forebrain caused impairments in hippocampal memory and synaptic plasticity, in parallel with specific reduction of NMDA receptor-mediated responses and in synaptic levels of NMDA receptors and α -CaMKII (Saura et al., 2004). Neurodegeneration of the cerebral cortex was accompanied by hyperphosphorylation of protein tau with increasing age in conditional PS1/PS2 knockout mice (Feng et al., 2004; Saura et al., 2004). Protection against hyperphosphorylation of Tau by presenilins was also evident in drosophila, as well as deduced from in vitro studies (Baki et al., 2004; Doglio et al., 2006). The rather different functions of presenilins in synaptic plasticity and cognition and in protection against neurodegeneration, raised the interesting hypothesis that EOFAD-linked mutations in PS1 and PS2 contribute to the pathogenesis of AD by a loss of function mechanism or by dominant negative effects (Koo and Kopan, 2004; Marjaux et al., 2004).

To address this hypothesis experimentally we examined whether mutant PS1 causes a dominant negative effect on either or both synaptic plasticity and Tau phosphorylation. Hereto we comparatively analyzed the in vivo repercussions of postnatal neuron-specific deficiency of PS1 and postnatal neuron-specific expression of mutant PS1[A246E] on synaptic plasticity and Tau phosphorylation. We demonstrate that both neuronal PS1 deficiency and expression of mutant PS1[A246E] affect synaptic plasticity, however in opposite directions, associated with opposite effects on the cellular and molecular correlates of synaptic plasticity, i.e. NMDAreceptor responses and NMDA-receptor subunit expression at the postsynaptic density. In addition, we demonstrate for the first time a presynaptic role for PS1 in vivo, that is not modulated by mutant PS1[A246E]. Finally we demonstrate that mutant PS1 increased Tau phosphorylation accompanied by decreased phosphorylation of GSK-3β at Ser-9. The presented data shed light on the mechanisms involved in the modulation of synaptic plasticity by PS1 and mutant PS1, demonstrating a presynaptic role and postsynaptic modulation of NMDA-receptor functioning.

2. Experimental procedures

2.1. Mice

The generation of the three types of PS1 transgenic mice that were analyzed here, was described previously: mice overexpressing wild-type PS1 (PS1/WT/7) or mutant

PS1[A246E] (PS1/Mut/2) (Dewachter et al., 2000; Herms et al., 2003; Moechars et al., 1999) and PS1(n–/–) mice with postnatal neuronal deletion of PS1 by Cre-recombinase mediated excission (Dewachter et al., 2002). Importantly, all transgenes, including the Cre recombinase, were under control of the same mouse thy1 gene promoter, warranting postnatal, neuron specific expression. All experimental procedures were performed in accordance with the regulations of, and authorized by the Ethical Commission for Animal Experimentation of the K.U.Leuven.

2.2. Electrophysiological measurements

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 NaHCO₃, 1.24 mM KH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgSO₄, and 10 mM glucose, saturated with 95% O2 and 5% CO2. Electrophysiological recordings were made with bipolar tungsten microelectrodes to stimulate Schaffer's collaterals. Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in CA1 with low resistance $(2M\Omega)$ glass microelectrodes filled with 2M NaCl. Test stimuli were 0.1-ms constant voltage pulses delivered every 30 s at an intensity sufficient to evoke an \sim 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 0.5 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. NMDAR responses were recorded in Mg²⁺-free ACSF in the presence of 20 µM CNQX. AMPAR responses were measured in the presence of D-APV 50 µM. AMPAR and NMDAR responses were measured at increasing stimulation intensity ranging from 2 V to 12 V.

2.3. Biochemical analysis

2.3.1. Biochemical analysis of postsynaptic densities:

Postsynaptic densities (PSDs) were isolated from mouse brains and separated from other synaptic elements based on insolubility in the detergent Triton X-100, using a standard protocol (Carlin et al., 1980; Herms et al., 1999) with minor modifications. Cerebrum of age- and sex-matched PS1 mutant transgenic mice and non-transgenic mice (n = 12 for each genotype) and PS1(n-/-) and control mice (n = 12 for each genotype) was used. Brains of 2 mice were pooled and homogenized in 8 ml of ice-cold 0.32 M sucrose containing protease inhibitors (Amersham) and phosphatase inhibitors (NaF, sodium ortho-vanadate, okadaic acid) using a glass-Teflon homogenizer. Protease and phosphatase inhibitors were included in all buffers. The resulting homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant was pelleted again at $9200 \times g$ for 15 min, and the resulting crude synaptosomal pellet was resuspended in buffer B (0.32 M sucrose and 1 mM HEPES), loaded onto a sucrose density gradient containing 15 ml each of 1.4 and 1.0 M sucrose, and centrifuged at $82,500 \times g$ for 60 min in a Beckman SW 28 rotor. The band between 1.4 and 1.0 sucrose was collected and diluted in buffer B to a volume of 10 ml. After adding an equal volume of 1% (v/v) Triton X-100, the suspension was stirred for 15 min at 4 °C and centrifuged at $32,800 \times g$ for 10 min. The resulting pellet was resuspended in buffer B and loaded on another sucrose density gradient containing 5 ml of 2.0 M sucrose, 3 ml of 1.5 M sucrose and 3 ml of 1.0 M sucrose. After centrifugation at $201,800 \times g$ for 120 min in the Beckman SW 40 rotor, the PSD fraction band between 1.5 and 2.0 M sucrose was collected, diluted in buffer B to a volume of 1.2 ml, mixed with an equal volume of 1% Triton X-100 in 150 mM KCl and pelleted at $113,500 \times g$ for 10 min. The resulting pellet containing PSDs was collected in 20 mM HEPES buffer. Western blotting was performed using anti-NR1C1 (Ab6484), anti-NR2B (Ab109), anti-NR2A (Ab108), anti-PSD-95 (Ab13552), anti-CaMKII alpha (phospho T286) (Ab2724) antibody (Abcam Ltd., Cambridge, UK), antic-Fos antibody (SC-52) (Santa Cruz Biotechnology) for detection of the respective proteins. Densitometric scanning of films and normalization were performed using a diluted sample series as described (Dewachter et al., 2000, 2002; Moechars et al., 1999).

2.3.2. Biochemical analysis of Tau phosphorylation

Cerebrum was dissected out and snap frozen in liquid nitrogen and homogenized in six volumes of homogenization buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 30 mM sodium fluoride, 2 mM sodium vanadate, 1 µM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, $5 \mu g/ml$ pepstatin, $5 \mu g/ml$ soy bean trypsin inhibitor) in a Potter-Elvejhem homogenizer with 10 up-and-down strokes of a Teflon pestle rotating at 700 rpm. Homogenates were diluted appropriately in sample buffer for SDS-PAGE on 8% polyacrylamide gels (Novex, San Diego, CA). Western blotting was performed as described previously (Dewachter et al., 2000) using as primary antibodies Tau-5 (Pharmingen, BD Biosciences, California, USA), AD2 (Bio-Rad, California, USA), anti-GSK-3β (Transduction Laboratories, BD Biosciences, California, USA) and anti-GSK-3ß ser9P (Cell Signalling, Massachusetts, USA). Quantitation was performed using densitometric scanning of films using a flatbed optical density scanner and dedicated software for analysis and measurement (Image Master; Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. Ultrastructural morphological analysis

Ultrastructural morphometric analysis was performed based on standard protocols (Pozzo-Miller et al., 1999). Mice were anesthetized and transcardially perfused with 0.9% NaCl for 2min followed by 4% paraformaldehyde and 0.05% glutaraldehyde in PBS for 5'. The brains were removed and placed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Sagittal sections were made at 300 µm thickness and stored in the same fixative till the day of processing. After rinsing in 0.1 M phosphate buffer pH 7.4 and 7.5% sucrose, sections were post-fixed (1 h) with 1% OsO4 in veronal acetate buffer pH 7.4 at 4 °C, rinsed in veronal acetate buffer pH 7.4 and 7% sucrose, stained en bloc with 0.5% uranyl acetate in veronal acetate buffer pH 5.2 (40 min). After a rinse in veronal acetate buffer pH 5.2 and dehydration in ethanol series, slices were rinsed in propylene oxide and flat-embedded in Embed 812. Ultrathin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Synaptic density was measured by counting synapses (i.e. postsynaptic densities) at a $8900 \times$ magnification (and confirmed at 21,000× magnification). The average length of the active zone was measured at $21,000 \times$ magnification. The parameters of the presynaptic terminal were measured at $54,000 \times$ magnification (i.e. area of the presynaptic terminal, length of the active zone in contact with the presynaptic terminal, number of reserve pool $(\sim 50 \text{ nm diameter})$ synaptic vesicles per terminal, and number of effectively and total docked vesicles). Only complete profiles of non-perforated asymmetric synapses on dendritic spines were photographed. Digital images were made with a transmission electron microscope (Philips EM10) at 80 kV using Analysis software. Synaptic vesicles were counted only when their plasma membranes were clearly defined for at least one-half their circumference. A total of 110 asymmetric synapses on CA1 dendritic spines from PS1(n-/-) mice, control mice, PS1[A246E] transgenic mice and non-transgenic mice were analyzed (three mice of each genotype). Quantitative analysis was performed blinded from the genotype. Digital image analysis was performed using NIH Image.

2.5. Behavioral analysis

2.5.1. Spatial memory test-object recognition test

Spatial learning in the Morris water maze and object recognition memory were performed essentially as described (Dewachter et al., 2002; Moechars et al., 1999; Rampon et al., 2000; Tang et al., 1999). Because FVB/N mice are not optimal for behavioral tests (Dewachter et al., 2002, and references therein), PS1 mutant hybrid F1 (FVB/NxC57/Bl6) mice were generated and crosses of FVB and C57Bl for mice with a neuronal deficiency of PS1. Age- and sexmatched mice of 4-6 months old were used for behavioral analysis. Contextual and cued fear conditioning: Fear conditioning experiments were performed in a standard fear conditioning chamber with a grid floor connected to a current shocker (Med. Associates, Inc., St. Albans, VT). The training consisted of a single trial; after a 60 s baseline period, mice were exposed to 3 tone (CS)-footshock (US) pairings (tone (CS), 30 s, 3000 Hz, 90 dB; footshock (US), 2 s, 0.5 mA; intertrial interval, 2 min; CS and US co-terminated

at the end of the training block). Ninety seconds after the last shock, mice were returned to their home-cages. After 24 h (or 3 days), contextual fear conditioning was assessed by returning the mice to the conditioning chamber during a 5 min period. For measuring cued fear conditioning the CS was presented (90 dB; 3000 Hz) during 3 min, 2 min after the mouse was placed in a novel chamber, created by covering the floor and all side-walls with white plexiglass. Freezing was quantified automatically using a video-based conditioned fear testing system, FreezeFrame (Actimetrics Software, Evanston, IL). Freezing was presented as the percent time spent freezing (time spent freezing/total time \times 100). All data are expressed as mean \pm S.E.M. (n = 12control; n = 9 PS1(n - l - l) mice; n = 12 non-transgenic mice; n = 10 PS1[A246E] mice) and analyzed by ANOVA followed by post hoc-Tukey tests.

3. Results

3.1. Synaptic plasticity in mutant PS1 and PS1(n-/-) mice

Hippocampal LTP at the Schaffer collaterals and CA1 pyramidal neurons was measured in brain slices derived form PS1(n-/-) mice and littermates, expressing the floxed PS1 gene but not Cre-recombinase, further referred to as control mice (Dewachter et al., 2002). LTP evoked by a strong tetanic stimulation, i.e. two trains of 100 Hz during 1 s separated by 20 s, of the Schaffer collaterals resulted in a significant decrease in potentiation in hippocampal slices from PS1(n-/-) mice compared to control mice (Fig. 1). The slope of fEPSP in PS1(n-/-) mice was restored progressively to control levels 2 h after the tetanic stimulus (Fig. 1A).



Fig. 1. Synaptic plasticity in PS1(n-/-) and PS1[A246E] mice. (A) The initial phase of long-term potentiation in CA1 region of hippocampus is impaired in PS1(n-/-) mice. The percentage of potentiation of field EPSPs recorded before and after tetanic stimulation of Schaffer's collaterals in brain slices from PS1(n-/-) and control mice are presented in panel A. Each data point shown is the mean \pm S.E.M. of results from eight individual mice of each genotype. A statistically significant decrease was evident in the initial phase of LTP (*p < 0.05; Student's *t*-test). Insets, examples of typical fEPSP recorded 15 min after HFS (traces b and d) superimposed on control trace (a and c) for control (left trace) and PS1(n-/-) mice (right trace). The final LTP after 2 h was not significantly different in PS1(n-/-) mice as compared to control mice. (B) Long-term potentiation is facilitated in CA1 region of PS1[A246E] mice as compared to non-transgenic and PS1[wt] transgenic mice. The percentage of potentiation of the slope of fEPSPs recorded before and after weak HFS stimulation, i.e. trains of 0.5 s at 100 Hz with 0.1-ms pulses, in PS1[A246E] transgenic mice, PS1[wt] transgenic mice and non-transgenic mice (*n* = 8 per genotype; *p < 0.05 Student's *t*-test) are presented in panel B. Insets, examples of typical fEPSP recorded 2 h after weak stimulation (traces b and d) superimposed on control trace (a and c) for non-transgenic mice (left) and PS1[A246E] mice (right). (C and D) Paired pulse facilitation is impaired in PS1(n-/-) mice, while not significantly changed in PS1[A246E] mice. (C) Paired pulse facilitation is impaired at interpulse-interval (IPI) of 50-ms in PS1(n-/-) as compared to control mice. A representative trace at IPI of 50 ms in PS1(n-/-) and control mice (C, right panel) and mean \pm S.E.M. are presented (p < 0.05 Student's *t*-test) (C, left panel). (D) Paired pulse facilitation was not different between PS1[A246E] mice and non-transgenic mice at IPI of 50 ms (D) nor at IPI of 25 ms, 75 ms and

LTP evoked by high frequency stimulation in hippocampal slices of PS1[A246E] mice, PS1[wt] mice and non-transgenic mice was not altered (results not shown). However, weak tetanic stimulation, i.e. one train of 0.1 ms pulses for 0.5 s at 100 Hz induced significantly stronger LTP in the hippocampus of PS1[A246E] as opposed to PS1[wt] mice and non-transgenic mice (p < 0.05). No significant differences in potentiation in PS1[wt] and non-transgenic mice were detected (Fig. 1B). Expression of the human PS1 protein was two-fold higher in PS1[wt] expressing mice as compared to PS1[A246E] (Dewachter et al., 2000), excluding increased expression levels as the cause of the facilitated LTP in brain of PS1[A246E] mice (Supplemental data, Table S2).

To study the molecular and cellular mechanisms involved in the modulation of LTP by mutant PS1[A246E] and neuronal deficiency of PS1, we analyzed different pre- and postsynaptic parameters. Paired-pulse facilitation (PPF), a widely used index of presynaptic function (Wang et al., 2004), was measured in CA1 of PS1(n-/-) and control littermates, using the ratio of the second and the first EPSP slopes at an interpulse interval (IPI) of 50 ms. PPF was significantly impaired in slices from PS1(n-/-) mice relative to control mice, i.e. $154 \pm 4\%$ in PS1(n-/-) mice and $179 \pm 6\%$ in control mice (p < 0.05) (Fig. 1C). PPF measured at IPI of 50 ms was not significantly different in PS1[A246E] compared to non-transgenic mice (Fig. 1D). Even at IPI of 25 ms, 75 ms and 100 ms no significant differences were measured between PS1[A246E] mice and non-transgenic mice (results not shown).

To further analyze the effects of neuronal PS1 deficiency on presynaptic functioning we measured synaptic fatigue (Huang et al., 2004; Dobrunz and Stevens, 1997). Measuring fEPSP during tetanic stimulation (100 Hz, 1 s) demonstrated a subtle but significant increase in synaptic fatigue in PS1(n-/-) mice as compared to control mice (Supplemental data S1B).

3.2. NMDA and AMPA responses in PS1(n-/-) and mutant PS1 mice

To analyze the effects of PS1 deficiency and mutant PS1[A246E] at the postsynaptic side, we next measured NMDA and AMPA receptor responses in the CA1 region of hippocampal slices of PS1(n-/-) and mutant PS1 mice. AMPAR responses displayed a tendency to decrease, only significant (p < 0.05) at stimulation intensity of 7 V, in PS1(n-/-) mice and were not changed in PS1[A246E] mice. Pharmacological isolation of NMDAR responses by addition of 20 μ M CNQX to the medium, revealed a significant decrease in NMDAR responses in CA1 of PS1(n-/-) mice, as opposed to increased NMDAR mediated responses in CA1 of mutant PS1 mice (Fig. 2). The

ratio of NMDAR/AMPAR mediated responses was significantly decreased in PS1(n-/-) mice, while increased in PS1[A246E] mice (Fig. 2). These results indicate that neuronal PS1 deficiency and neuronal expression of mutant PS1 affected NMDA-receptor function in opposite directions.

3.3. Biochemical analysis of NMDA and AMPA receptors in brain of mutant PS1 and PS1(n-/-) mice

To define the underlying molecular parameters of altered NMDA-responses due to PS1 deficiency and mutant PS1, we analyzed the protein levels and the phosphorylation state of selected components in the postsynaptic density (PSD). PSD is structurally essential for synaptic transmission and synaptic plasticity (Kennedy, 1997). PSD fractions were purified from mouse brain by a protocol that is based on its resistance to solubilization by Triton X-100, consisting essentially of two sequential sucrose density gradients (Carlin et al., 1980; Herms et al., 1999; Walikonis et al., 2000; also see Section 2). Quantitative analysis of the AMPA-receptors GluR1 subunit, the NMDA-receptor subunits NR1, NR2A and NR2B and the phosphorylated α -CaMKII kinase, was performed on isolated PSD fractions from mice with the different genotypes, and compared after normalization for PSD-95 levels.

The levels of NR1, NR2B and phosphorylated α -CaMKII were significantly lower in PSD preparations from brain of PS1(n-/-) mice relative to control mice (Fig. 3) (p < 0.05). In contrast, PSD fractions isolated from brain of mutant PS1[A246E] mice contained significantly more NR1, NR2B and phosphorylated α -CaMKII than those from non-transgenic mice (Fig. 3) (p < 0.05).

NMDA-dependent Ca²⁺ influx triggers a biochemical cascade that also activates immediate early genes (IEGs) like c-fos (Fleischmann et al., 2003). Moreover, c-fos is induced following a variety of learning experiences in mice (Miyamoto, 2006; Tischmeyer and Grimm, 1999) and c-fos deficiency impairs memory (Fleischmann et al., 2003). Measurement of nuclear c-fos levels revealed a significant decrease in brain extracts of PS1(n-/-) mice compared to control mice, as opposed to a significant increase in PS1[A246E] mice relative to non-transgenic mice (Fig. 4).

3.4. Ultrastructural analysis of synaptic morphology in CA1 region of mutant PS1 and PS1(n-/-) mice

The structural correlate of synaptic changes induced by PS1 deficiency and by mutant PS1[A246E] were explored by morphometric ultrastructural analysis of excitatory synapses in the CA1 region of the hippocampus. Parameters measured were synapse density, length of the active zone, number of docked vesicles per length of active zone, and density of reserve pool vesicles. Glutamatergic excitatory synapses on CA1 dendritic spines were identified by established criteria: (i) presynaptic profiles containing small (~50 nm diameter), round and clear synaptic vesicles; (ii) prominent electron-dense postsynaptic densities, characteristic of type-I



Fig. 2. NMDA and AMPA receptor-mediated synaptic responses in PS1(n-/-) and PS1[A246E] mice. (A and B) Plot of AMPAR-responses and NMDAR-responses by the appropriate use of specific blockers D-APV (50 μ M) and CNQX (20 μ M), respectively, measured as extracellular fEPSP induced in PS1[A246E] mice (right panel) and PS1(n-/-) mice (left panel) by stimulation of the Schaffer collaterals with increasing current intensities. This demonstrates a significant decrease in NMDAR responses in CA1 in PS1(n-/-) mice (p < 0.05), as opposed to a significant increase in PS1[A246E] mice (p < 0.05). (C) Calculated ratios of NMDAR/AMPAR-responses at increasing stimulation intensities of the Schaffer collaterals. Plots depict mean \pm S.E.M. (n = 5/n = 5 PS1(n - / -)/control mice; n = 6/n = 6 PS1[A246E]/non-transgenic mice) and demonstrate an increased ratio NMDAR/AMPAR PS1[A246E] mutant mice, as opposed to a decreased ratio of NMDAR/AMPAR in PS1(n - / -) (p < 0.05).

asymmetric synaptic junctions; and (iii) postsynaptic dendritic spines, identified by their lack of microtubules and mitochondria, continuity with a dendritic shaft, and overall size and shape (Peters et al., 1991). Quantitation of synaptic density and the length of the active zone revealed no significant differences in PS1(n-/-) mice or PS1[A246E] mice (Fig. 5).

The distribution of synaptic vesicles was measured in two distinct and mutually exclusive pools within presynaptic terminals at CA1 excitatory synapses, i.e. docked vesicles and reserve pool of vesicles. Docked vesicles were defined as being located less than one vesicle diameter (\sim 50 nm) from the active zone (Pozzo-Miller et al., 1999; Tartaglia et al., 2001). Reserve pool vesicles were identified as located within the presynaptic terminals but outside the docked vesicle region, corresponding to a reserve pool of quanta. The number of effectively docked vesicles, i.e. in contact with the presynaptic membrane, per active zone



Fig. 3. Biochemical analysis of proteins at the postsynaptic density demonstrates decreased postsynaptic concentration of NR1/NR2B subunits and phosphorylated α -CaMKII (T286) in PS1(n-/-) mice as opposed to increased concentrations in PS1[A246E] mice. Semi-quantitative Western blotting analysis of proteins present in postsynaptic density preparations. Representative Western blots immunostained for AMPA-receptor (GluR1 subunit), NMDA receptor subunits NR2B, NR2A and NR1, α -CaMKII (phospho T286) and PSD-95 are presented. Representative samples of control mice (lanes 1 and 2; denoted "control") and PS1(n-/-) mice (lanes 3 and 4; denoted "PS1(n-/-)") are presented in the left panel. Samples of non-transgenic mice (lanes 1 and 2; denoted "Non-tg") and samples of PS1[A246E] transgenic mice (lanes 3 and 4; denoted "PS1 mut") are presented in the right panel. Quantitation was done on 12 mice per genotype (brains of 2 mice were pooled for preparation of 1 PSD sample) and using a dilution series of sample. Western blotting analysis was performed in triplicate. Data are presented as mean \pm S.E.M. (p < 0.05).



Fig. 4. Nuclear c-Fos concentrations are decreased in PS1(n-/-) mice, in contrast to increased concentrations in PS1[A246E] mice. (A) Western blotting analysis of c-Fos in nuclear extracts of PS1(n-/-) mice (lanes 1 and 3; denoted "PS1(n-/-)") and control mice (lanes 2 and 4; denoted "control"). Semi-quantitative analysis reveals decreased nuclear c-Fos levels in PS1(n-/-) mice as compared to control mice. Histograms present relative expression of nuclear c-Fos compared to non-transgenic mice (%OD) mean \pm S.E.M. (p < 0.05). (B) Western blotting analysis of c-Fos in nuclear extracts of PS1[wt] mice (lanes 1 and 4; denoted "PS1 wt"), PS1[A246E] mice (lanes 2 and 5; denoted "PS1 mut") and non-transgenic mice (lanes 3 and 6; denoted "Non-tg"). Semi-quantitative analysis demonstrates increased nuclear c-Fos levels in PS1[A246E] mice as compared to PS1[wt] and non-transgenic mice.



Effectively docked vesicles/ 100nm active zone

Fig. 5. Ultrastructural morphometric analysis of synapses in hippocampal CA1 region in PS1(n-/-) and PS1[A246E] mice reveals decreased number of effectively docked vesicles and reserve pool of vesicles in synapses of CA1 in PS1(n-/-) mice. (A) Quantitative analyses of synaptic spines in stratum radiatum of hippocampal CA1 region. The histograms plot: (i) number of effectively docked synaptic vesicles per 100 nm active zone length, (ii) the number of reserve pool vesicles normalized per synaptic terminal area (RV/ μ m²), (iii) the length of the active zone (nm), (iv) synaptic vesicle density in stratum radiatum of hippocampal CA1 region expressed as number of synapses per 100 μ m². The left panels present the values of PS1(n-/-) mice and control mice, the right panels present the values for PS1[A246E] mice and non-transgenic mice. Data are presented as mean ± S.E.M. (*p < 0.05). B. Representative electron micrographs of spine synapses at 21,000× and 54,000× magnification as used for ultrastructural morphometric analyses of spines in PS1(n-/-) and PS1[A246E] mice. The large white arrow indicates a docked synaptic vesicle, small black arrows indicate synaptic vesicles of the reserve pool (upper panel). The large white arrow indicates the postsynaptic density, which is bound by the two black arrows (lower panel).

length (DV/ μ m) was significantly lower in PS1(n-/-) mice than in control mice (p < 0.05; n = 28) (Fig. 5). The number of total docked vesicles was similarly significantly lower in PS1(n-/-) mice, i.e. 17.7 ± 0.6 vesicles/ μ m compared to 20.2 ± 0.5 vesicles/µm in control mice (p < 0.05; n = 28). Furthermore, the reserve pool of vesicles per unit presynaptic terminal was significant decreased by neuron-specific inactivation of PS1, i.e. 244.6 ± 11.3 vesicles/µm² in PS1(n-/-)

mice relative to 297.9 ± 15.9 vesicles/ μ m² in control mice (p < 0.05; n = 28) (Fig. 5). In contrast, neuronal expression of mutant PS1[A246E] did neither affect the number of effectively and total docked vesicles, nor the number of reserve pool vesicles (Fig. 5). The data combined with the functional data, demonstrate a presynaptic role of PS1, which is unaffected in mutant PS1[A246E] mice.

3.5. Cognitive functioning of mutant PS1 and PS1(n-/-) mice

Modulation of cognitive function by PS1 deficiency and mutant PS1[A246E] was assessed in three different tasks involving hippocampal plasticity, i.e. spatial navigation task, object recognition task, and cued and contextual fear conditioning task. We previously reported unchanged cognitive performance in the Morris water maze of PS1[A246E] mice compared to non-transgenic mice (Schneider et al., 2001). Here we observed normal learning and memory in the Morris water maze of the PS1(n-/-) mice (results not shown).

Cognition of PS1[A246E] mice and PS1(n-/-) mice was further assessed in a paradigm of nonspatial visual recognition memory, i.e. the novel object recognition task that is known to depend on hippocampal activity (Rampon et al., 2000; Tang et al., 1999). After training to familiarize all mice with a given object, they were tested for retention

by confronting them with a novel object, next to and in addition to the familiar one. Testing after 3 h demonstrated no statistically significant differences in retention in PS1(n-/-) mice nor in PS1[A246E] mice as compared to control mice (results not shown).

Emotional learning and memory of PS1[A246E] mice and PS1(n-/-) mice was assessed in contextual and cued fear conditioning paradigms. Cued fear conditioning is dependent on intact amygdala, while contextual fear conditioning requires both hippocampus and amygdala (Maren and Holt, 2000; Maren and Quirk, 2004; Rumpel et al., 2005). Surprisingly, 24 h after training the PS1[A246E] mice displayed a significantly augmented contextual memory (p<0.05) (Fig. 6), while PS1(n-/-) mice were normal in this respect. On the other hand, PS1(n-/-) mice when exposed to the conditioned context 3 days after training (p<0.05) (Fig. 6). Cued conditioning was not significantly changed in either PS1(n-/-) or in PS1[A246E] mice (results not shown).

The cognitive behavioral analysis demonstrated that neither mutant PS1 nor neuronal deficiency of PS1, affected the cognitive performance in spatial navigation or in object recognition tasks. However, the significant change in contextual fear conditioning reveals a subtle impairment in the PS1(n-/-) mice, while in PS1[A246E] mice cognitive performance after 1 day was improved.



Fig. 6. Cued and contextual fear conditioning in PS1(n-/-) and PS1[A246E] mice. (A) Percentage of freezing during a 5 min context test in PS1(n-/-) as compared to control mice 1 day (upper panel) or 3 days (lower panel) after training (p < 0.05). (B) Percentage of freezing responses during a 5 min context test in non-transgenic and PS1[A246E] mice 1 day (upper panel) or 3 days (lower panel) after training (p < 0.05). (B) Contextual fear conditioning is improved in PS1[A246E] mice 1 day after training but not 3 days after training, in contrast to subtle impairments in PS1(n-/-) mice. Cued fear conditioning, spatial navigation in the water maze and object recognition memory were not affected in PS1[n-/-) and PS1[A246E] mice (results not shown).



Fig. 7. Increased Tau phosphorylation and decreased GSK-3 β inactivation in brain of PS1[A246E] mice. Semi-quantitative Western blotting analysis revealed increased Tau phosphorylation at Ser-396 and Ser-404, and more Tau proteins migrating with slower electrophoretic mobility, corresponding to hyperphosphorylated Tau isoforms, in brain of PS1[A246E] (lane 3 and 4, denoted "PS1 mut", grey bars, n = 6) as compared to non-transgenic mice (lanes 1 and 2, denoted "Non-Tg", black bars, n = 5) (panel A), while not in PS1(n-/-) mice (control mice: lanes 1 and 2, denoted "Control", black bars, n = 5) (PS1(n-/-)", grey bars, n = 5) (panel B). Western blotting analysis was performed with monoclonal antibody AD-2, detecting protein Tau phosphorylated at Ser-396 and Ser-404, and with Tau-5 antibody, detecting all phosphorylated and non-phosphorylated isoforms of protein Tau. Western blotting analysis of total GSK-3 β and GSK-3 β phosphorylated on Ser-9, revealed decreased concentrations of GSK-3 β phosphorylated at Ser-9, while total concentrations of GSK-3 β are unchanged in brain of PS1[A246E] (grey bars, n = 6) as compared to non-transgenic mice (black bars, n = 5) (panel A). In brain of PS1(n-/-) mice no changes in Tau phosphorylation at Ser-396 and Ser-404 or GSK-3 β phosphorylation at Ser-396 and Ser-404 or GSK-3 β phosphorylation at Ser-396 and Ser-404 or GSK-3 β phosphorylation at Ser-9 were detected as compared to control mice (panel B) (control mice: lanes 1 and 2, denoted "Control", black bars, n = 5) (PS1(n-/-) mice: lane 3 and 4, denoted "PS1(n-/-)", grey bars, n = 5). Histograms present mean \pm S.E.M. (*p < 0.05).

3.6. Mutant PS1 increases Tau phosphorylation correlating with decreased GSK- 3β inactivation

Since mutant PS1[A246E] did not exert dominant negative effects on synaptic plasticity and cognition, we explored other parameters that have been put forward by the pathological process, in first instance phosphorylation of protein tau. The monoclonal antibody AD-2 detects protein Tau phosphorylated at Ser-396 and Ser-404, a pathologically relevant phosphorylation site (Buee-Scherrer et al., 1996) and also phosphorylated by GSK-3 β in mouse brain in vivo (Spittaels et al., 2000).

Analysis by Western blotting revealed increased phosphorylation of protein tau in old PS1[A246E] mice compared to non-transgenic mice (Fig. 7). In contrast, neuronal deficiency of PS1 even in PS1(n-/-) mice as old as 22 months, did not reveal differences in tau phosphorylation at the AD2 epitope, potentially due to redundancy of PS2 in adult brain. Western blotting with the Tau-5 antibody that detects all phosphorylated and non-phosphorylated isoforms of protein Tau, revealed more Tau proteins migrating with slower electrophoretic mobility, known to correspond to hyperphosphorylated tau isoforms, in brain of PS1[A246E] mice but not of PS1(n-/-) mice (Fig. 7).

Increased phosphorylation of protein Tau correlated with decreased phosphorylation of GSK-3 β on Ser-9 in brain extracts of PS1[A246E] mice, while total GSK-3 β protein levels remained unchanged (Fig. 7). The data support the hypothesis that mutant PS1 affected the phosphorylation of protein tau at Ser-394–Ser-404 by activating GSK-3 β in adult mouse brain.

4. Discussion

FAD-linked mutants of PS1 have been proposed to exert pathogenic effects not only by increasing the production of the longest and less soluble A β 42, but also by independent loss-of-function effects (Marjaux et al., 2004; Saura et al., 2004). This attractive hypothesis is based on observations that

implicate presenilins in normal physiological mechanisms that vary widely from synaptic plasticity to phosphorylation of protein tau (Baki et al., 2004; Doglio et al., 2006; Feng et al., 2004; Herms et al., 2003; Saura et al., 2004; Yoo et al., 2000). In this study we comparatively analyzed synaptic plasticity in mice with a neuronal deficiency of PS1 and mice expressing mutant PS1[A246E]. In depth analysis of pre- and postsynaptic parameters directly involved in synaptic plasticity, revealed a presynaptic function of presenilin1 reflected in pre-synaptic vesicle pools and docked synaptic vesicles, which was not affected by mutant PS1. Moreover, NMDA-receptor mediated electrophysiological responses were affected in opposite directions by mutant PS1 and by neuronal deficiency of PS1, closely paralleled by similar changes in the levels of NR1/NR2B receptor subunits at the postsynaptic density.

In this report we analyzed the effects of mutant presenilin1[A246E] and presenilin1 on synaptic plasticity as well as its molecular and cellular correlates. The initial phase of long-term potentiation in Schaffer collaterals was impaired in PS1(n-/-) mice. This correlated with decreased NMDAreceptor responses in CA1 region and lower levels of NR1 and NR2B receptor subunits in the postsynaptic density, as well as postsynaptic phosphorylated CaMKII and nuclear c-Fos. Previous studies have reported unaltered PPF and LTP in forebrain specific PS1 knockout mice (Feng et al., 2001; Yu et al., 2001). Genuine differences may result from conditional inactivation of PS1 by Cre recombinase expression under control of either the α -CaMKII gene promoter or the murine thyI gene promoter, although both steer expression postnatally and neuron specific. Subtle defects in spatial navigation were detected previously (Yu et al., 2001) while not here in PS1(n-/-) mice. We believe that the actual stimulation protocols and the experimental conditions can reveal subtle defects in synaptic plasticity and cognition. Our data demonstrate a normal physiological function for neuronal presenilin1 per se in synaptic plasticity and cognition. The synaptic phenotype in PS1(n-/-) mice is qualitatively similar but quantitatively less pronounced than in PS1/PS2dKO mice (Saura et al., 2004), revealing additional redundancy or overlap of PS1 and PS2 in their regulation of synaptic plasticity.

We further demonstrate that compared to PS1(n-/-)mice, synaptic plasticity is oppositely affected in PS1[A246E] mice. These mice exhibited enhanced LTP, correlating with increased NMDA-receptor responses in hippocampal slices. Concentrations of NR1/NR2B NMDA receptor subunits were increased in the postsynaptic densities, with concomitantly increased levels of activated CaMKII translocated to the postsynaptic density and nuclear c-Fos. It must be noted that we analyzed the effect of mutant PS1 in PS1[A246E] transgenic mice, which might raise the criticism that the demonstrated effects are merely due to "overexpression". However, overexpression of wild-type PS1 at two-fold higher levels than mutant PS1, did not affect LTP, indicating that not the overexpression, but the mutant itself is responsible for the effects on LTP, and the postsynaptic parameters. In addition, facilitated LTP in the hippocampal CA1 region is a consistent finding in mice expressing mutant PS1, including PS1 mutant knock-in mice, demonstrating again that not overexpression but the mutation itself facilitates LTP (Barrow et al., 2000; Oddo et al., 2003; Parent et al., 1999).

Hippocampal LTP, particularly in CA1, is known to correlate with cognitive performance in hippocampus-dependent memory tasks (Malleret et al., 2001; Moechars et al., 1999; Saura et al., 2004; Tang et al., 1999) although also exceptions have been reported. We observed subtle changes in cognitive performance: the PS1[A246E] mice performed better in the contextual fear test 1 day after training, in contrast to a subtle impairment in PS1(n-/-) mice, while both genotypes behaved normally in tasks for object recognition and spatial memory. Contradictory results have been reported on cognition of mutant PS1 mice, ranging from subtly decreased performance, over no effects, to improved performance (Huang et al., 2003; Janus et al., 2000; Wang et al., 2004). Our data are in line with a previous report demonstrating that mutant PS1 improved short-term cognitive performance but was followed by increased deterioration (Vaucher et al., 2002).

The role of NR2B in synaptic plasticity and cognition has been intensively studied during the last years. Switching synaptic NR2B-containing NMDA-Rs that bind CaMKII with high affinity with those containing NR2A, a subunit with low affinity for CaMKII, dramatically reduces LTP (Barria and Malinow, 2005). In contrast, studies with pharmacological inhibitors of NR2A and NR2B receptors proposed that activation of NR2A receptors solely controls induction of LTP (Liu et al., 2004). This view was however experimentally challenged (Barria and Malinow, 2005; Berberich et al., 2005) and is at odds with in vivo data in mice overexpressing NR2B or with a genetic blockage of NR2B (Tang et al., 1999; Wong et al., 2002; Zhao et al., 2005). Genetic blockage of NR2B impaired LTP and contextual fear memory (Zhao et al., 2005), while overexpression of NR2B enhanced activation of NMDA receptors, facilitated long-term potentiation at 10-100 Hz and increased performance in various cognitive tasks (Malleret et al., 2001). The role of NR2B in enhanced short-term cognitive performance is further demonstrated in transgenic mice overexpressing KIF17, the transporter of the NR2B subunit with upregulated NR2B expression (Wong et al., 2002). Our current data in mutant PS1 transgenic mice are completely in line with the hypothesis that increased NR2B expression enhances activation of NMDA receptors, facilitates long-term potentiation and increases short-term cognitive performance.

Besides a role for PS1 at the postsynaptic side, we also demonstrate, to our knowledge for the first time in vivo, the direct involvement of PS1 in presynaptic functioning. Ultrastructural morphometric analysis revealed a reduction of the reserve pool of synaptic vesicles and a decrease in the number of docked vesicles per active zone in brain of PS1(n-/-) mice, the latter parameter being a morphological correlate of the readily releasable pool (Schikorski and Stevens, 2001). We further observed a significant decrease in paired-pulse facilitation and increased synaptic fatigue, indicating impaired presynaptic functioning. These data suggest that PS1 deficiency might result in decreased reserve pool of synaptic vesicles and increased release probability. This is in line with the in vitro demonstration of increased mEPSC frequency, and higher release probability in primary hippocampal PS1 deficient neurons (Parent et al., 2005). The exact mechanisms underlying the presynaptic deficits in PS1(n-/-) mice need further analysis, but may relate at the molecular level to proteolytic processing of substrates, to calcium ion homeostasis and/or to axonal vesicle and protein transport.

FAD mutant PS1 knock-in mice display increased phosphorylation of protein tau by a putative GSK-3 β dependent mechanism (Tanemura et al., 2006). We here report that the FAD linked PS1[A246E] mutation activates GSK-3 β and increases phosphorylation of protein tau in vivo. Phosphorylation of protein tau was decreased in double PS1/PS2 conditional knockout mice, in Drosophila and in cellular models (Baki et al., 2004; Feng et al., 2004; Saura et al., 2004). The mechanism whereby increased phosphorylation of tau, is brought about by mutant PS1 remains to be explored. In any case, the effect of PS1 deficiency on synaptic vesicles, as discussed in the previous paragraph, cannot be ascribed to interference of phosphorylated tau with axonal transport of pre-synaptic vesicles.

In summary, our data demonstrate that neuronal PS1 deficiency and neuronal expression of PS1[A246E] both affect synaptic plasticity, but in opposite directions on many important parameters of synaptic plasticity. Our data demonstrate that the more aggressive onset and pathogenesis of mutant PS1 EOFAD cannot be explained by a dominant negative effect on synaptic plasticity, but rather might relate to a dominant negative effect on protection against Tau phosphorylation or by rendering synapses more responsive or more vulnerable to toxic insult. Our data yield fundamental insight in the role of PS1 and mutant PS1 in the modulation of tau phosphorylation and synaptic plasticity in vivo, as well as its molecular and cellular correlates. Further studies are needed to unravel their role in detail and will help to design more appropriate therapeutic strategies targeting PS1 as the essential subunit of γ -secretase. These need to consider additional functions of PS1, besides its evident direct activity in the processing of APP and generation of amyloid peptides.

Disclosure statement

There are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence their work. No author's institution has contracts relating to this research through which it or any other organization may stand to gain financially now or in the future. There are no other agreements of authors of their institutions that could be seen as involving a financial interest in this work. The data contained in the manuscript being submitted have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging. All authors have reviewed the contents of the manuscript being submitted, approved of its contents and validated the accuracy of the data. All experiments with animals were performed in accordance with the regulations of, and authorized by the Ethical Commission for Animal Experimentation of the K.U.Leuven.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging. 2006.11.019.

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