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Presenilins regulate calcium homeostasis and presynaptic function via ryanodine receptors in hippocampal neurons

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Presenilin (PS) plays a central role in the pathogenesis of Alzheimer's disease, and loss of PS causes progressive memory impairment and age-related neurodegeneration in the mouse cerebral cortex. In hippocampal neurons, PS is essential for neurotransmitter release, NMDA receptor-mediated responses, and long-term potentiation. PS is also involved in the regulation of calcium homeostasis, although the precise site of its action is less clear. Here we investigate the mechanism by which PS regulates synaptic function and calcium homeostasis using acute hippocampal slices from *PS* conditional knockout mice and primary cultured postnatal hippocampal neurons, in which PS is inducibly inactivated. Using two different calcium probes, Fura-2 and Mag–Fura-2, we found that inactivation of PS in primary hippocampal neurons does not affect calcium concentration in the endoplasmic reticulum. Rather, in the absence of PS, levels of ryanodine receptor (RyR) are reduced in the hippocampus, measured by Western analysis and radioligand binding assay, although the mRNA expression is unaffected. RyR-mediated function is also impaired, as indicated by reduced RyR agonist-induced calcium release from the ER and RyR-mediated synaptic responses in the absence of PS. Furthermore, knockdown of RyR expression in wild-type hippocampal neurons by two independent shRNAs to levels comparable with the RyR protein reduction in *PS*-deficient hippocampal neurons mimics the defects exhibited in calcium homeostasis and presynaptic function. Collectively, our findings show that PS regulates calcium homeostasis and synaptic function via RyR and suggest that disruption of intracellular calcium homeostasis may be an early pathogenic event leading to presynaptic dysfunction in Alzheimer's disease.

ER calcium | caffeine

Mutations in the *presenilin* (*PS*) genes account for ∼90% of causative mutations in familial Alzheimer's disease (AD), highlighting the importance of PS in the pathogenesis of AD. We previously reported that presenilins play essential roles in the regulation of long-term potentiation, short-term plasticity and neurotransmitter release (1–4). Interestingly, presynaptic defects caused by loss of presynaptic PS can be mimicked by depletion of calcium in the endoplasmic reticulum (ER), suggesting that disrupted intracellular calcium homeostasis may underlie these presynaptic defects (2). Although large numbers of reports have indicated an involvement of PS in the regulation of intracellular calcium homeostasis, the precise site of its action is less clear (4, 5).

The ER is a major source of intracellular calcium and is present in both axonal and dendritic compartments (6). Cytosolic calcium is pumped into the ER by sarco-ER calcium ATPase (SERCA), and calcium release from the ER into the cytosol can be mediated through either the ryanodine receptor (RyR) or the inositol 1,4,5 trisphosphate receptor (IP_3R). Blockade of the RyR but not the IP_3R mimics and occludes the presynaptic defects in hippocampal slices of CA3-*PS* conditional double knockout (cDKO) mice (2). In the absence of PS, calcium-induced calcium release (CICR) mediated by RyR is impaired in cultured primary hippocampal neurons (2). It has also been reported that IP_3R-mediated calcium release was reduced in fibroblasts lacking either PS1 alone or both presenilins (7) and that overexpression of wild-type PS1 potentiates IP_3R-mediated calcium release in *Xenopus* oocytes (8). More recently, PS was proposed as the calcium leak channel on the ER, based on the observations that reconstituted PS protein formed divalent cation-permeable ion channels in bilayer membranes and that calcium release from the ER was increased in fibroblasts lacking presenilins (9). However, this notion has been challenged (10, 11), and reduced calcium concentration in the ER has also been reported using the same immortalized *PS*-null fibroblasts (12). Thus, despite the importance of PS in the regulation of intracellular calcium homeostasis, the site at which presenilins act is controversial. The immortalized *PS*-null fibroblasts, which exhibit general transcriptional defects independent of PS (13), are widely used in these studies and may contribute to the differences in the outcomes. It is therefore critically important to address how PS regulates intracellular calcium homeostasis in primary cells, preferably in a physiologically relevant setting coupled with electrophysiological analysis to elucidate its role in synaptic function and calcium regulation.

In the current study, we use primary hippocampal cultures and acute hippocampal slices to determine whether loss of PS affects ER calcium concentration and how PS regulates presynaptic function. Using two calcium probes (Fura-2 and Mag–Fura-2), we found that calcium levels in the ER are normal in *PS* cDKO hippocampal neurons. Interestingly, levels of RyR protein expression are reduced in the hippocampus of postnatal forebrain-*PS* (FB-*PS*) cDKO mice and in primary cultured hippocampal neurons, although the mRNA levels are unaffected. Radioligand binding assay using [^{3}H]ryanodine further confirmed the reduction of functional RyRs in the hippocampus of FB-*PS* cDKO mice. RyR function is impaired in *PS* cDKO hippocampal neurons, as indicated by the reduced calcium release from the ER induced by RyR agonists, caffeine and 4-chloro-m-cresol (4-CmC), and the smaller caffeine-induced synaptic potentiation in CA3-*PS* cDKO mice. Knockdown of RyR expression in hippocampal neurons by two shRNAs to a level comparable with *PS* cDKO neurons mimics the reduced CICR and the impaired synaptic facilitation observed in *PS* cDKO neurons. Together, these findings show that PS regulates ER calcium homeostasis and presynaptic function through RyRs.

Results

Loss of PS Does Not Affect ER Calcium Concentration in Primary Cultured Hippocampal Neurons. Loss of PS has been reported to increase (9) or decrease (12) ER calcium concentration in

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immortalized *PS*-null fibroblasts, by serving as an ER calcium leak channel or interacting with SERCA to inhibit its activity, respectively. We sought to address this issue using inducible *PS* cDKO hippocampal neurons, which were derived from postnatal pups to circumvent the embryonic developmental requirement of PS (2, 13–17). We first measured ER calcium concentration ($[Ca^{2+}]_{ER}$) indirectly using thapsigargin, which inhibits SERCA activity, resulting in depletion of ER calcium due to the lack of calcium refilling. As we reported (2), cytosolic $[Ca^{2+}]$ under basal conditions is normal in the absence of PS (Fig. 1 *A* and *B*). In the absence of extracellular Ca^{2+}, application of thapsigargin (1 μM) triggers Ca^{2+} increase in the cytosol due to Ca^{2+} release from the ER. Both the peak and the area of the thapsigargin-induced Ca^{2+} response curves are similar in *PS* cDKO and control neurons (Fig. 1 *A* and *B*), suggesting normal $[Ca^{2+}]$ in the ER.

To measure $[Ca^{2+}]_{ER}$ more directly, we loaded cultured neurons with a low-affinity Ca^{2+} indicator, Mag–Fura-2, which is routinely used to monitor ER calcium (18). Brief exposure of Mag–Fura-2AM preloaded neurons to digitonin (10 μM), which permeabilizes the neurons, results in the loss of the Mag–Fura-2 signal in the cytosol (Fig. 1*C*). The $[Ca^{2+}]_{ER}$ is measured by the increase in the F340/F380 ratio of the remaining Mag–Fura-2 signal (Fig. 1*C*). Ionomycin treatment, which induces calcium release from the ER, indeed leads to gradual decreases of calcium signal, indicating that calcium signal at the platform reflects its $[Ca^{2+}]_{ER}$. We found that the $[Ca^{2+}]$ at the platform is similar between control and *PS* cDKO neurons (Fig. 1*C*), providing further support that $[Ca^{2+}]_{ER}$ is normal in primary cultured hippocampal neurons lacking PS.

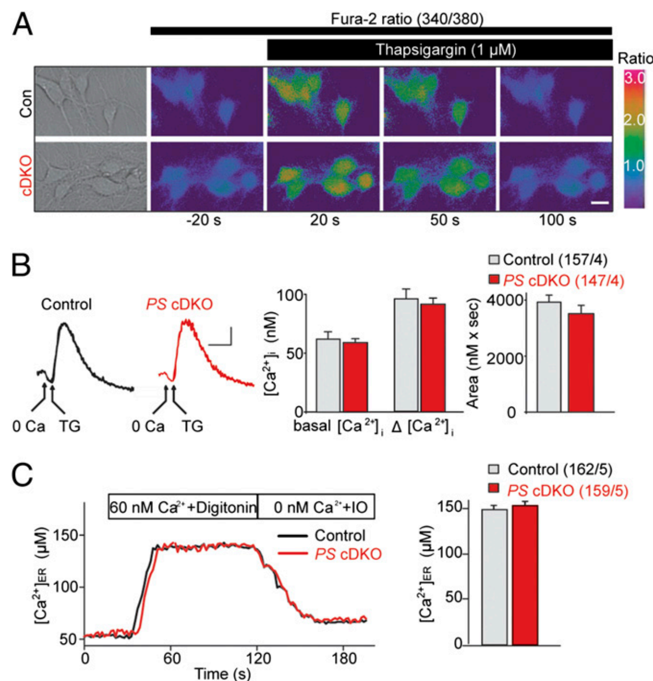

Fig. 1. Normal $[Ca^{2+}]_{ER}$ in primary postnatal *PS* cDKO hippocampal neurons. (*A*) Representative calcium images show thapsigargin (1 μM)-induced Ca^{2+} responses in control and *PS* cDKO neurons. (Scale bar: 20 μm.) (*B*) Normal thapsigargin-induced responses in cultured *PS* cDKO hippocampal neurons. (*Left*) Representative traces of thapsigargin (1 μM)-induced Ca^{2+} responses in control and *PS* cDKO neurons. (Scale bar: 25 s, 25 nM.) (*Right*) Normal basal cytosolic $[Ca^{2+}]$ and thapsigargin-induced Ca^{2+} responses (peak amplitude and total area) in *PS* cDKO neurons. (*C*) Normal $[Ca^{2+}]_{ER}$ measured directly by Mag–Fura-2 in *PS* cDKO neurons. (*Left*) Representative traces of $[Ca^{2+}]$ recorded by Mag-Fura-2 in control and *PS* cDKO neurons. The ratio of Mag–Fura-2 signals excited at 340 and 380 nm is converted into $[Ca^{2+}]$. At the beginning of the experiment, $[Ca^{2+}]$ is relative low, reflecting mostly cytosolic origin of the signal. Permealization with digitonin initiates an increase in $[Ca^{2+}]$, and it reaches a platform after the washout is complete. The value at the platform represents the $[Ca^{2+}]_{ER}$. (*Right*) Quantitative analysis shows normal $[Ca^{2+}]_{ER}$ measured by the F340/F380 ratio of Mag–Fura-2 signals in *PS* cDKO neurons. All data represent mean ± SEM. The values in parentheses indicate the number of hippocampal neurons (*Left*) and the number of independent experiments (*Right*).

Reduced Levels of RyR Proteins in the Hippocampus of *PS* cDKO Mice. We previously reported that CICR is impaired in *PS* cDKO hippocampal neurons (2). The normal $[Ca^{2+}]_{ER}$ suggests that this reduction in CICR is not due to reduced calcium levels in the ER. To determine whether the reduction of RyR-mediated CICR in the absence of PS is caused by reduced expression and function of RyR, we performed a number of analyses. There are three isoforms of RyRs (RyR1-3) encoded by three different genes, all of which are expressed in the brain (19), and four subunits coalesced to a stable homo-tetramer form a calcium channel with a single ion pore in the center (20). We first measured mRNA levels of the three RyR isoforms using quantitative RT-PCR in the hippocampus of FB-*PS* cDKO and control mice at 2 mo of age, and found that mRNA levels of each RyR isoform are unchanged in the absence of PS (Fig. 2*A*). However, Western analysis showed that levels of total RyRs are decreased in both *PS* cDKO hippocampi (Fig. 2*B*) and cultured hippocampal neurons (see Fig. 5*B*). Using an RyR2-specific antibody, we found levels of RyR2 are also reduced in the hippocampus of FB-*PS* cDKO mice (Fig. 2*B*).

To determine whether levels of functional RyRs are similarly affected in the absence of PS, we performed radioligand binding assays with $[^3H]$-labeled ryanodine, which is widely used as a monitor of channel activity because high-affinity ryanodine binding occurs preferentially to the open state of the channel (21). $[^3H]$Ryanodine binding is significantly reduced in hippocampal areas CA1 and CA3 of FB-*PS* cDKO mice (Fig. 2*C*), although the differences are much smaller compared with total RyR levels (Fig. 2*B*). The $[^3H]$ signal is specific as it can be eliminated when excess unlabeled ryanodine is used (Fig. 2*C*). These results provided further support that RyR levels are reduced in the absence of PS.

Impaired RyR-Mediated Calcium Release in *PS* cDKO Hippocampal Neurons. To determine whether the reduction of RyR indeed causes impairment of RyR-mediated calcium release, we used caffeine (10 mM), a widely used agonist of RyRs (22), to activate RyR function. Cytosolic $[Ca^{2+}]$ was monitored by Fura-2 signals. Consistent with our previous results, before stimulation with caffeine, there was no difference in basal cytosolic $[Ca^{2+}]$ between control and cultured *PS* cDKO hippocampal neurons (Fig. 3 *A* and *B*). However, application of caffeine induced a smaller Ca^{2+} increase in cultured *PS* cDKO hippocampal neurons (Fig. 3 *A* and *B*), indicating RyR-mediated calcium release is reduced in the absence of PS.

Although caffeine is a widely used RyR agonist to induce calcium release from ER calcium stores, it also has additional targets, such as phosphodiesterase and purinergic receptors (23). We therefore used another RyR agonist, 4-CmC (0.5 mM), which is specific for RyR at concentrations below 1 mM (24) and has been used to study RyR function in a variety of cells including hippocampal neurons (25, 26). We found that the peak of 4-CmC–induced calcium response is reduced in *PS* cDKO neurons (Fig. S1), providing further evidence for an impairment of RyR function in the absence of PS.

To test whether the reduced caffeine-induced Ca^{2+} response in *PS* cDKO neurons can be rescued by exogenously introduced PS1, we infected *PS* cDKO and control hippocampal neurons with a lentivirus expressing wild-type human PS1 (hPS1) at day in vitro (DIV) 10. Western analysis of the culture lysate collected at DIV14 showed no accumulation of APP C-terminal fragments (CTFs) in *PS* cDKO neurons expressing hPS1 (Fig. S2*A*), indicating that exogenous hPS1 restored the lack of γ-secretase activity in *PS* cDKO neurons. Calcium imaging performed at DIV14 showed that the reduction of caffeine-induced Ca^{2+} response is also restored in *PS* cDKO neurons expressing hPS1 (Fig. S2*B*).

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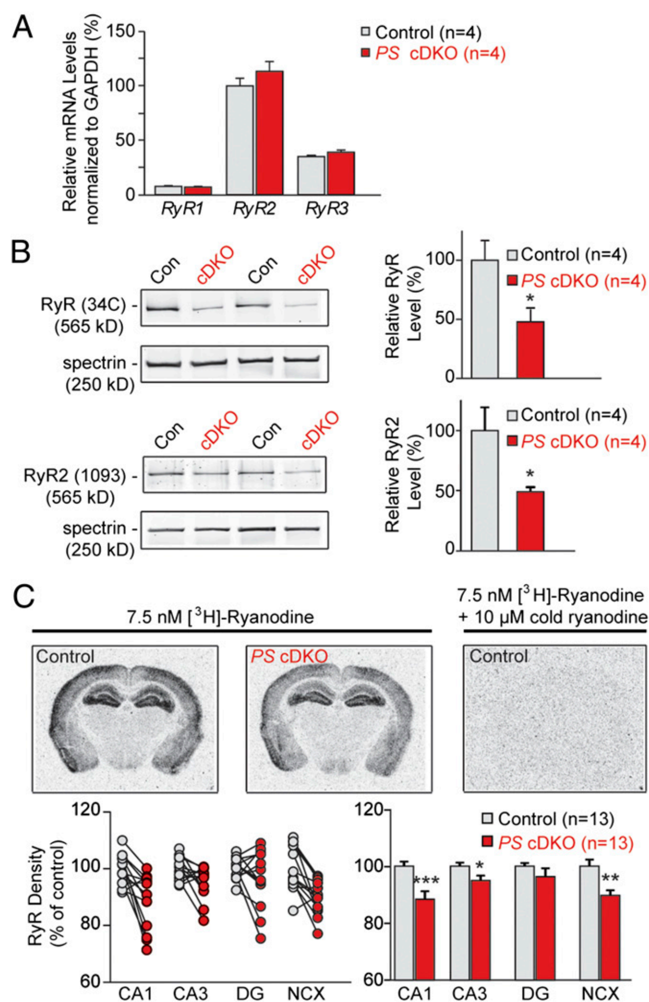

Fig. 2 Decreased levels of RyR proteins but not mRNAs in the hippocampus of *PS* cDKO mice. (*A*) Quantitative RT-PCR showing normal relative expression levels of *RyR1, RyR2* and *RyR3* mRNAs, after normalized to *GAPDH* mRNA, in the hippocampus of FB-*PS* cDKO mice. (*B*) Protein levels of total RyRs or RyR2 are reduced in the FB-*PS* cDKO hippocampus at 2 mo of age. The anti-RyR antibody, 34C (Abcam, ab2868), recognizes all three isoforms of RyRs. The anti-RyR2 antibody 1093 is specific for RyR2 (47). Total protein lysates (100 μg) were loaded in each lane. (*C*) Reduced [^{3}H]ryanodine-binding in hippocampal subregions of FB-*PS* cDKO mice at 2–3 mo of age. (*Top*) Representative coronal sections show autoradiographic localization of [^{3}H]ryanodine-binding sites in the neocortex and hippocampus of control and *PS* cDKO mice. The brain sections were processed in the presence of [^{3}H]ryanodine (7.5 nM) alone or in the presence of [^{3}H]ryanodine and unlabeled ryanodine (10 μM). (*Bottom Left*) Dot plots comparing RyR density in hippocampal subregions in pairs of control and *PS* cDKO mice that were processed together. (*Bottom Right*) Summarized data of RyR density in CA1, CA3, dentate gyrus (DG), or neocortex (NCX) subregions in control and *PS* cDKO mice. All data represent mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The values in parentheses indicate the number of mice used in each experiment.

To determine whether the RyR levels are specifically affected by the loss of PS or whether this could be a compensatory change due to reduced SERCA activity or expression, we measured the levels of SERCA and IP$_3$R. Western analysis showed normal levels of SERCA2, which is the predominant SERCA isoform in the brain (27), in the *PS* cDKO hippocampus and *PS* cDKO hippocampal neuronal cultures (Fig. S3*A*). Consistent with our earlier findings showing the lack of effect in calcium homeostasis upon IP$_3$R blockade in *PS* cDKO hippocampal neurons (2), levels of IP$_3$R1, the major isoform of IP$_3$R in the brain (28), are also normal in the hippocampus of *PS* cDKO mice and *PS* cDKO hippocampal cultures (Fig. S3*B*). To measure SERCA activity directly, we monitored Ca^{2+} dynamics in the ER following SERCA activation and inhibition using Mag–Fura-2 in individual digitonin-permeablized neurons. We found no difference in the filling rate, Ca^{2+} loading and leak rate, suggesting that SERCA activity in *PS* cDKO neurons is unaffected (Fig. S4).

Impaired RyR-Mediated Synaptic Function in *PS* cDKO Mice. To determine the effect of PS inactivation on RyR-mediated synaptic function, we tested whether activation of RyRs could differentially affect synaptic response in the presence and absence of PS. Application of caffeine (10 mM) induced robust enhancement in field excitatory postsynaptic potentials (fEPSPs) in both genotypes but the enhancement is smaller in CA3-*PS* cDKO mice, compared with control mice (Fig. 4*A*). Because caffeine blocks presynaptic adenosine A1 receptors (23), which inhibit synaptic transmission when they are activated (29), we treated the hippocampal slices with a specific RyR inhibitor dantrolene (10 μM) to dissect out the effect of RyR on caffeine-induced enhancement in synaptic response. Dantrolene treatment reduces caffeine-induced synaptic enhancement in control but not in CA3-*PS* cDKO mice (Fig. 4*B*), indicating that the additional enhancement of synaptic responses in control mice is mediated through RyR. Similar results were obtained in FB-*PS* cDKO mice, but no differences were found in CA1-*PS* cDKO mice compared with the control (Fig. S5). These results suggest that in the absence of presynaptic PS, RyR-mediated synaptic function is impaired.

Knockdown of RyR in Cultured Hippocampal Neurons Mimics the Impaired Calcium Homeostasis and Presynaptic Deficits of *PS* cDKO Neurons. To determine whether the calcium and presynaptic defects in *PS* cDKO hippocampal neurons are due to reduced levels of RyR proteins, we designed two independent shRNAs to knockdown RyR expression in hippocampal neuronal culture. At DIV10 we introduced the shRNA targeting all three isoforms of RyRs by lentivirus (Fig. 5*A*). The reduction of RyR protein levels begins 2 d after transduction. Four days following lentiviral infection, levels of RyRs are reduced to 46.6 ± 6.6% of control, which is similar to the reduction of RyR levels in *PS* cDKO hippocampal cultures. Therefore, in the following calcium imaging experiments and electrophysiological recordings, we used the culture 4 d after we introduced the lentivirus to knockdown RyR (DIV14). We found

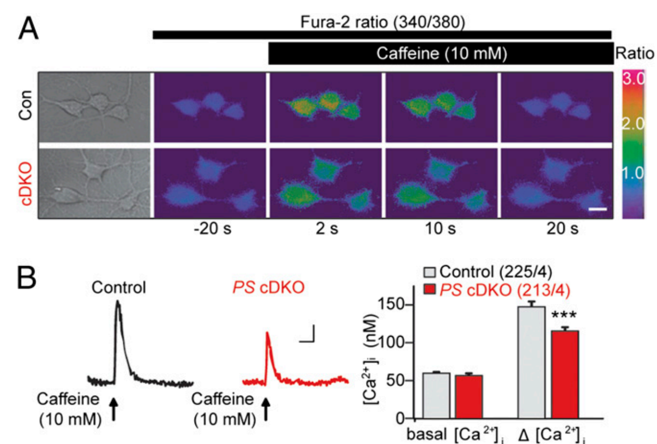

Fig. 3. Reduced calcium release from the ER calcium store induced by RyR agonist, caffeine, in *PS* cDKO neurons. (*A*) Representative calcium images show caffeine (10 mM)-induced Ca^{2+} responses in control and *PS* cDKO neurons. (Scale bar: 20 μm.) (*B*) Reduced caffeine-induced Ca^{2+} responses in *PS* cDKO neurons. (*Left*) Representative traces of caffeine-induced Ca^{2+} responses. (Scale bar: 10 s, 50 nM.) (*Right*) Amplitude of caffeine-induced Ca^{2+} responses is reduced in *PS* cDKO neurons. All data represent mean ± SEM. ***$P < 0.001$. The values in parentheses indicate the number of neurons (*Left*) and independent experiments (*Right*) used in the study.

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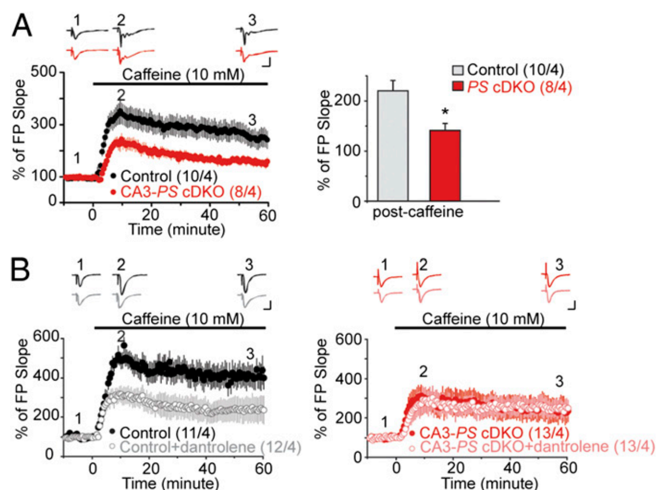

Fig. 4. Impairment of caffeine-induced synaptic potentiation in CA3-*PS* cDKO mice. (*A*) Effect of caffeine (10 mM) on the fEPSP slopes in CA3-*PS* cDKO mice (filled red circles) compared with controls (filled black circles). The representative traces of fEPSP at $t = -5$ min (1), 5 min (2), and 50 min (3) are shown. (Scale bar: 20 ms, 1 mV.) Bar graph shows the averages of 5 consecutive responses 60 min after application of caffeine. (*B*) Effect of caffeine (10 mM) on the fEPSP slopes in hippocampal slices of control mice in the absence (filled black circles) or presence (open gray circles) of dantrolene, and effect of caffeine (10 mM) on the fEPSP slopes in CA3-*PS* cDKO mice in the absence (filled red circles) or presence (open pink circles) of dantrolene. Representative traces of fEPSP at $t = -5$ min (1), 5 min (2), and 50 min (3) are shown. (Scale bar: 20 ms, 1 mV.) *$P < 0.05$. All data represent mean ± SEM. The number of slices (*Left*) and mice (*Right*) used in each experiment is indicated in parenthesis.

~50% reduction of RyRs in RyR shRNA-infected neurons at DIV14 (Fig. 5*B*). Similar to *PS* cDKO neurons (2), caffeine- and KCl (80 mM)-induced calcium increases in the cytosol are also reduced (Fig. 5 *C* and *D*).

We previously reported that synaptic facilitation is impaired in acute hippocampal slices derived from *PS* cDKO mice (2). To determine whether synaptic frequency facilitation in cultured *PS* cDKO hippocampal neurons is similarly affected, and whether *RyR* knockdown (KD) hippocampal neurons share the same presynaptic defects, we compared synaptic facilitation in both *PS* cDKO and *RyR* KD hippocampal neurons. Synaptic facilitation is similarly reduced in *RyR* KD (Fig. 5*E*) and *PS* cDKO neurons (Fig. 5*F*). Another shRNA targeting specifically for RyR2 yielded similar results in reduction of RyR expression, and impairment of caffeine- and KCl-induced cytosolic calcium increases as well as synaptic facilitation (Fig. S6). These results show that reduced RyR expression recapitulates the CICR and presynaptic deficits in *PS* cDKO hippocampal neurons, suggesting that PS regulates calcium homeostasis and presynaptic function via RyR.

Discussion

Presenilins have been reported to regulate calcium homeostasis in the ER, and dysregulation of intracellular calcium has been implicated in the pathogenesis of AD (4, 5). In the current study, we take advantage of our inducible *PS* cDKO primary hippocampal cultures and acute hippocampal slices of *PS* cDKO mice to investigate the role of PS in calcium homeostasis and synaptic function. To our great surprise, we found that ER calcium concentration measured indirectly by Fura-2 or directly by Mag–Fura-2 is unaffected in cultured hippocampal neurons lacking PS (Fig. 1). Rather, levels of RyR evaluated by Western blotting and radioligand binding assay are reduced in the absence of PS (Fig. 2). The reduced RyR expression is associated with impaired RyR function, as indicated by decreases in RyR agonist-induced calcium release (Fig. 3) and RyR-mediated synaptic responses (Fig. 4). Furthermore, reduction of RyR expression by two independent shRNAs mimics the impairment of caffeine-induced calcium release and presynaptic short-term plasticity observed in *PS* cDKO hippocampal neurons (Fig. 5). Thus, presenilins control ER calcium homeostasis and presynaptic short-term plasticity through the regulation of RyR expression and function.

PS Does Not Regulate $[Ca^{2+}]_{ER}$. PS has been implicated in the regulation of intracellular Ca^{2+} homeostasis, but the precise site(s) of its action remains controversial (4). For example, it was proposed that PS holoproteins act as the passive ER calcium leak channel, based primarily on larger ionomycin-sensitive Ca^{2+} pool in immortalized *PS* DKO mouse embryonic fibroblasts (MEFs) (9, 30). However, using ER-targeted calcium probes, including Mag–Fura-2, another group failed to find increases in Ca^{2+} stores in the ER using the same immortalized MEFs; rather, they found no difference in ER Ca^{2+} dynamics between the immortalized *PS* DKO MEFs and the DKO MEFs retrovirally transfected to express wild-type PS1 (11). Loss of PS in primary B cells derived from B-cell–specific *PS* cDKO mice does not affect ER calcium dynamics either (11). In contrast to these findings, *PS* DKO MEFs have also been reported to display reduced ER calcium levels, associated with either decreased SERCA activity (12) or increased IP_3R1 expression (31), although increased IP_3R1 expression is not associated with diminished Ca^{2+} in the ER in another study (11).

The basis of these contradictory results is less clear. To address the role of PS in the regulation of ER calcium homeostasis and synaptic function, we chose a physiologically more relevant preparation, primary hippocampal neurons, coupled with electrophysiological analysis to determine whether altered intracellular Ca^{2+} homeostasis underlies synaptic dysfunction observed in the hippocampus of *PS* cDKO mice (1–3). We evaluated ER Ca^{2+} in primary *PS* cDKO hippocampal cultures, which recapitulate the synaptic defects observed in acute hippocampal slices from *PS* cDKO mice (2). Using two independent Ca^{2+} indicators and large numbers of hippocampal neurons (n = 147–162 per genotype), we found that the $[Ca^{2+}]_{ER}$ is unchanged in *PS* cDKO neurons (Fig. 1). We measured ER Ca^{2+} content either directly using Mag–Fura-2 or indirectly using Fura-2 for cytosolic Ca^{2+} rise induced by thapsigargin. These results are consistent with earlier findings showing normal ER Ca^{2+} dynamics in *PS* DKO MEFs (11). We also found no changes in $[Ca^{2+}]_{ER}$ in primary *PS1* KO MEFs (Fig. S7) and immortalized *PS* DKO MEFs (Fig. S8) using Fura-2 to measure thapsigargin-induced Ca^{2+} increases in the cytosol. These results demonstrate that loss of PS1 or both PSs does not affect ER calcium concentration. Thus, the defects in CICR and presynaptic function observed in *PS* cDKO neurons are not caused by reduced ER $[Ca^{2+}]$.

Loss of PS Reduces RyR Expression and Function. Although $[Ca^{2+}]_{ER}$ is normal in *PS* cDKO hippocampal neurons, RyR-mediated Ca^{2+} release from the ER induced by RyR agonists, caffeine or 4-CmC, is reduced in *PS* cDKO hippocampal neurons (Fig. 3 and Fig. S1). The impaired RyR function in the absence of PS is consistent with our earlier findings showing that blockade of RyR using two independent antagonists mimics and occludes the presynaptic defects in acute hippocampal slices of *PS* cDKO mice (2). The impairment of RyR function is associated with reduced levels (~50%) of total RyR and RyR2, as measured by Western analysis of *PS* cDKO hippocampi and cultured hippocampal neurons (Fig. 2*B*). Furthermore, the reduced level of RyR protein in *PS* cDKO hippocampal neurons has also been confirmed by $[^3H]$ryanodine binding assay on brain sections (Fig. 2*C*), although the difference is smaller compared with Western result. $[^3H]$Ryanodine-binding assay is widely used to monitor channel activity because high-affinity ryanodine binding occurs preferentially to the open state of the channel (21). Thus, the reduction of functional RyRs in hippocampal neurons lacking PS is smaller than that of total RyRs. Consistent with previous reports (6, 32, 33), RyRs are more abundant in the hippocampal area CA3 and the dentate gyrus than the CA1 area. Furthermore, the reduction of RyR is unlikely to be a compensatory change due to reduced SERCA activity or expression,

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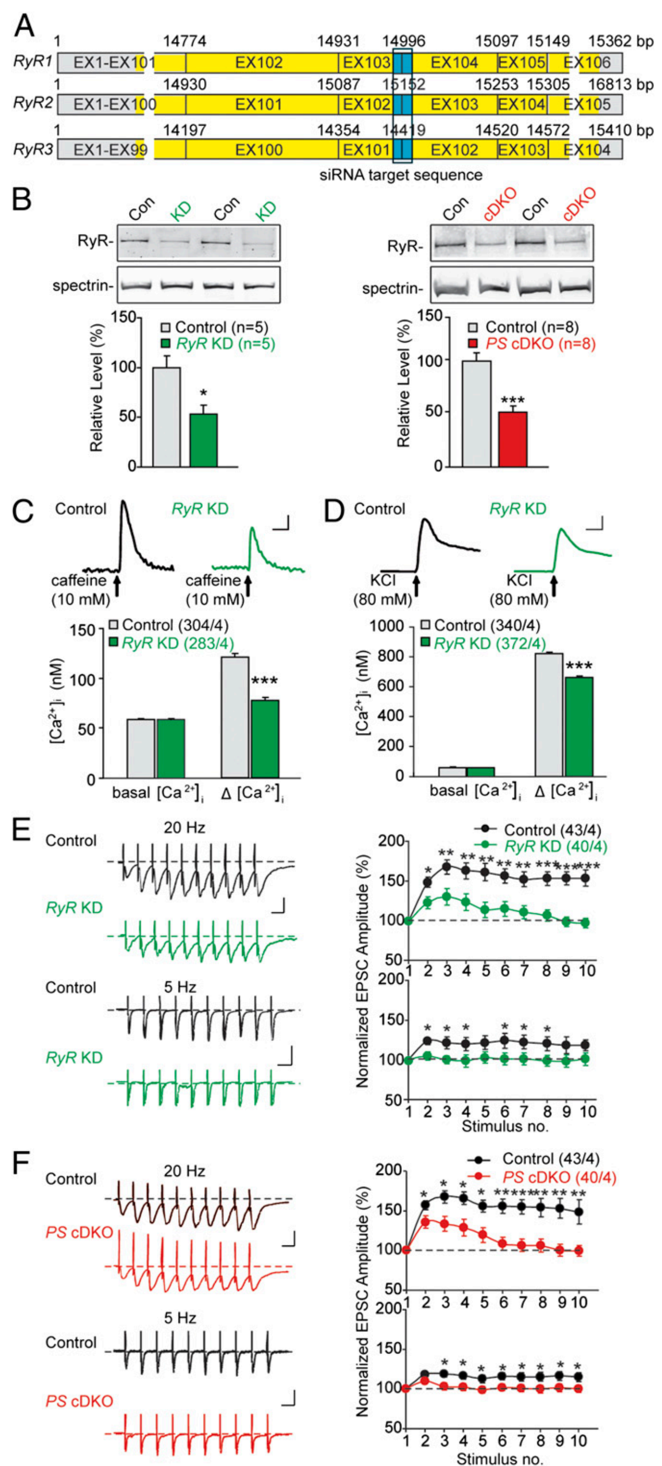

Fig. 5. Knockdown of RyR expression mimics the impaired calcium homeostasis and presynaptic function in *PS* cDKO neurons. (*A*) Schematic diagram showing the location of the shRNA targeting sequence for all three isoforms of RyR. (*B*) Comparison of RyR protein levels in *RyR* KD and *PS* cDKO cultures. (*Left*) Reduced RyR protein levels in hippocampal cultures 4 d after the lentivirus containing the shRNA sequence targeting all three isoforms of RyR is introduced to the hippocampal cultures. (*Right*) Reduced RyR protein levels in *PS* cDKO cultures. All samples are collected at DIV14. (*C*) Caffeine-induced calcium response is reduced in *RyR* KD neurons. (*Upper*) Representative traces of caffeine (10 mM)-induced Ca^{2+} responses. (Scale bar: 5 s, 25 nM). (*Lower*) Summarized amplitude of caffeine-induced Ca^{2+} responses in control and *RyR* KD neurons. (*D*) High potassium-induced Ca^{2+} response is reduced in *RyR* KD neurons. (*Upper*) Representative traces of KCl (80 mM)-induced Ca^{2+} responses. (Scale bar: 5 s, 200 nM.) (*Lower*) Summarized amplitude of KCl-induced Ca^{2+} responses in control and *RyR* KD neurons. (*E*) Synaptic facilitation elicited by stimulus trains of indicated frequencies is reduced in *RyR* KD neurons. [Scale bars: 50 ms, 50 pA (*Upper*); 200 ms, 50 pA (*Lower*).] (*F*) Synaptic facilitation elicited by stimulus trains of indicated frequencies is reduced in *PS* cDKO neurons. [Scale bars: 50 ms, 50 pA (*Upper*); 200 ms, 50 pA (*Lower*).] All data represent mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. The number of neurons (*Left*) and independent experiments (*Right*) used in the study are indicated in parenthesis.

as SERCA activity measured by filling rate of ER calcium and SERCA levels are similar between *PS* cDKO and control hippocampal neurons (Fig. S4). Furthermore, SERCA activity, ER calcium levels and calcium leak from the ER are also normal in *RyR* knockdown neurons (~50% reduction of RyR; Fig. S9). Thus, the reduction of RyR levels in *PS* cDKO mice is unlikely due to compensatory mechanisms in response to reduced SERCA activity/expression.

PS does not appear to regulate RyR expression at the transcriptional level, as the mRNA level of each of the RyR isoforms is unchanged in the hippocampus of *PS* cDKO mice. It is unclear how PS is involved in maintaining the level of RyRs, and whether other γ-secretase components are also required. Because both presenilins and RyRs are abundantly localized in the membrane of the ER, it is conceivable that the presence of PS is required for the stability of RyRs. Future studies will be needed to elucidate whether PS regulates the abundance of RyR protein in a γ-secretase–dependent or –independent manner.

PS and RyR in ER Calcium Homeostasis and Presynaptic Function. Our previous analysis of hippocampal CA3- or CA1-*PS* cDKO mice revealed that presynaptic PS plays a selective role in neurotransmitter release and long-term potentiation (LTP), and that inhibition of RyR function mimics and occludes the effects of PS inactivation in intracellular calcium homeostasis and presynaptic dysfunction (2). In the current study, we further showed that loss of PS reduces RyR expression and impairs RyR function in mediating calcium release and synaptic potentiation. The reduced RyR expression in *PS* cDKO neurons likely underlies the defects in CICR and presynaptic function, as knockdown of RyR expression by RNAi recapitulates these functional deficits.

The presence of RyR and ryanodine-sensitive Ca^{2+} stores in presynaptic terminals of various central synapses has been reported extensively (6, 34–38). For example, blockade of RyR and depletion of ER Ca^{2+} stores results in reduction in presynaptic Ca^{2+} transient and paired-pulse facilitation in CA3 pyramidal neurons (37), and caffeine induces presynaptic LTP independent of NMDAR activation or increases of postsynaptic calcium (39). Our findings showing that reduced RyR expression by shRNA leads to decreases in CICR and synaptic facilitation provide further support for the importance of RyR in presynaptic function. Indeed, 50% reduction of RyR in *PS* cDKO mice was sufficient to eliminate RyR-mediated synaptic potentiation (Fig. 4), and the effect of RyR reduction (~50%) by shRNA or PS inactivation on synaptic facilitation (Fig. 5 and Fig. S6) is similar to that of RyR blockade by ryanodine or dantrolene (2), further highlighting the importance of RyR in the regulation of synaptic function. We found that ~50% reduction of RyR by shRNA or PS inactivation does not affect the steady state $[Ca^{2+}]_{ER}$ or refill and leak rate (Fig. 1 and Figs. S4 and S9), but under this condition RyR activation-mediated Ca^{2+} release is affected (Figs. 3 and 5 and Figs. S1 and S6). These results suggest that under stimulated conditions RyR-mediated function is sensitive to reduced RyR levels. The impairment of RyR-mediated synaptic potentiation in FB- and CA3-*PS* cDKO mice but not in CA1-*PS* cDKO mice indicates that presynaptic PS is required for maintaining RyR-mediated presynaptic function. The dissection of RyR function in the presynaptic and postsynaptic terminals will await future development of cell type-specific cKO of RyRs.

RyR has been implicated in the pathogenesis of Alzheimer's disease. For example, [^{3}H]ryanodine binding and RyR2 protein levels are significantly reduced specifically in brain regions that are vulnerable to AD pathogenesis, such as the temporal and entorhinal cortex as well as the subiculum (40). Furthermore, caffeine intake has been associated with a significantly lower risk for AD and better cognitive performance among the elderly (41–45). The role of RyRs in AD pathogenesis can be further addressed by development of *RyR* cKO mice, and it would be worthwhile to pursue whether reversal of impaired RyR function could be a therapeutic strategy for the treatment of AD.

Materials and Methods

Calcium Imaging. Hippocampal neurons were loaded with Fura-2AM or Mag–Fura-2AM (5 μM, 45 min at 37 °C; Molecular Probes), and imaged with a Leica DMI6000 Microscope with 40× lens (numerical aperture 0.75) as described (2).

Electrophysiological Analysis. Acute hippocampal slices (400 μm) were prepared and recorded as described in ref. 2. Whole-cell patch recordings from cultured hippocampal neurons at ∼DIV14 were performed at room temperature using a Multiclamp 700B amplifier (Molecular Device) with pCLAMP acquisition software as described (2). All experiments were performed in a genotype blind manner.

RNAi Knockdown of RyR. Lentiviral shRNA constructs were made using the pLLX vector, which contains the U6 promoter for driving shRNA expression and the EGFP cDNA under the control of the Ubiquitin-C promoter (46). The targeting sequences for RyR and RyR2 are 5′ACATGGAGACCAAGTGCTT and 5′GGAAAGAAGTCGATGGCAT, respectively.

All experiments involving mice followed a protocol approved by Harvard Center for Animal Resources and Comparative Medicine. Full methods and associated references can be found in *SI Materials and Methods*.

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