Presenilin 1 increases association with synaptotagmin 1 during normal aging

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Abstract

Presenilin 1 (PS1), the catalytic component of gamma secretase, associates with synaptotagmin 1 (Syt-1). This interaction is decreased in the brains of patients with sporadic Alzheimer’s disease. However, it remains unclear how this interaction changes during normal aging. Because aging is a risk factor for Alzheimer’s disease, we sought to identify changes in PS1 and Syt-1 association during aging in primary neurons in vitro and mouse brain sections ex vivo. We also tested the effect of aging on the calcium dependence of the interaction by treating neurons aged in vitro with KCl. We found that PS1 and Syt-1 increase their association with age, an effect that is more robust in neuronal processes than cell bodies. Treatment with KCl triggered the interaction in both young and old neurons. Baseline calcium levels and calcium influx in response to KCl treatment were significantly higher in older neurons, which can partially explain the increase in PS1/Syt-1 binding with age. These results suggest a compensatory mechanism during normal aging to offset detrimental age-associated effects.

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by memory impairment and synaptic dysfunction. The pathological hallmarks of AD are amyloid plaques, neurofibrillary tangles, as well as neuronal and synaptic loss. Synaptic dysfunction and reduced synaptic density strongly correlate with memory impairment (Dickson et al., 1995; Fitting et al., 2013; Martins et al., 2017; Terry et al., 1991), and synaptic loss surpasses neuronal loss in AD (Davies et al., 1987). Synaptotagmin 1 (Syt-1) is an integral synaptic vesicle protein that acts as a direct calcium sensor (Stevens et al., 2003; Südhof, 2012; Sörensen et al., 2003; Xu et al., 2009) and together with other synaptic proteins is responsible for synaptic vesicle exocytosis and endocytosis (Yao et al., 2012; Zhou et al., 2017). Syt-1 is therefore one of the crucial proteins involved in regulating neuronal functioning at the level of the synapse. Whereas Syt-1 levels are decreased in AD brains because of synaptic loss (Zoltowska et al., 2017), it was recently reported that Syt-1 is elevated in the cerebrospinal fluid of patients with AD and thus may serve as a disease biomarker (Öhrfelt et al., 2016). This makes Syt-1 a compelling target for further investigation within the context of AD.

We have previously found that calcium influx triggers Syt-1 binding to presenilin 1 (PS1) (Kuzuya et al., 2016). The latter is the catalytic component of gamma secretase, an enzyme involved in cleaving the amyloid precursor protein to produce amyloid-beta (Aβ) peptides (Haass and De Strooper, 1999; Kuzuya et al., 2016; Wolfe et al., 1999). Syt-1 knockdown induces a “pathogenic” conformational shift of PS1 and an increase in the Aβ 42/40 ratio, suggesting that Syt-1 may play some modulatory role in the generation of synaptic Aβ peptides (Kuzuya et al., 2016). Furthermore, targeted inhibition of PS1/Syt-1 binding in neurons in vitro impairs exocytosis and neurotransmitter release and results in the accumulation of intracellular Aβ peptides (Zoltowska et al., 2017). These findings suggest that the PS1/Syt-1 interaction is important for synaptic upkeep by modulating both synaptic vesicle cycling and Aβ levels.
Aging is the major risk factor for developing AD (Fjell et al., 2014; Guerreiro and Bras, 2015; Niccoli and Partridge, 2012). However, it is unknown whether the PS1/Syt-1 interaction is affected by normal aging. To better elucidate the physiological relevance of the interaction between PS1 and Syt-1, it is important to understand the association of these two proteins throughout normal aging.

The present study investigates the interaction between PS1 and Syt-1 during normal aging and explores calcium's effects on PS1/Syt-1 association in aged neurons. The interaction was tested using both PS1/Syt-1 complex coimmunoprecipitation and fluorescence lifetime imaging microscopy (FLIM) in intact neurons. We found that association between the two proteins was significantly higher in older compared with younger neurons as determined by analyzing the PS1/Syt-1 interaction in primary neurons in vitro and in hippocampal neurons in mouse brains. The increased baseline levels of intracellular calcium and higher influx of calcium after KCl treatment in older neurons may underlie the observed increased association between PS1 and Syt-1 during aging. Because the PS1/Syt-1 interaction has a beneficial effect by protecting both PS1 conformation and Syt-1 functioning (Zolotowska et al., 2017), these results suggest that increased Syt-1 binding to PS1 during normal aging may have a compensatory protective function. Given that the PS1/Syt-1 interaction is decreased in the brains of patients with sporadic AD, fostering the proteins' association may be exploited therapeutically.

2. Materials and methods

2.1. Cell culture

Primary neuron cultures were obtained from embryos of CD1 pregnant female mice at gestation day 14–17 (Charles River Laboratories, Wilmington, MA). Cortical and hippocampal neurons were extracted using a Papain Dissociation Kit (Worthington, Lakewood, NJ) and plated onto tissue culture dishes and slides coated with poly-D-lysine (Millipore, Burlington, MA). Cells were seeded at 0.25–1.6 × 10⁵ cells per cm² on plastic or glass bottom slides (MatTek Corporation, Ashland, MA) or on plates or dishes (Corning Inc., New York, NY). Cells were maintained in an incubator at 37 °C with 5% CO₂ in culture medium containing neurobasal medium (Invitrogen, Carlsbad, CA) with 2% B27 supplement (Gibco, Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 1% Glutamax (Invitrogen). Medium was replenished on a weekly basis. Neurons at 8–28 days in vitro (DIV) were used for subsequent immunocytochemistry and coimmunoprecipitation experiments. Before collecting neurons for immunoprecipitation or FLIM, neurons were treated with either 50 mM KCl or vehicle in neurobasal medium for 15 minutes at 37 °C.

2.2. Mouse tissue

C57BL/6 mice at 5 months or 23–24 months of age were used (Charles River Laboratories). Each group contained three mice. Mice were housed in a light-dark cycle, in a temperature- and humidity-controlled facility with ad libitum food and water. Mice were euthanized with CO₂ and perfused immediately after with ice-cold phosphate-buffered saline and 4% paraformaldehyde (PFA). Brain tissue was then post-fixed for 28 hours in 4% PFA at 4 °C and cut using a freezing microtome (Leica SM 2000R, Bannockburn, IL) into sagittal or coronal sections (30 μm) and stored in 30% glycerol solution at −20 °C. All experiments involving mice were approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital using guidelines from the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Immunoprecipitation and Western blotting

Primary neurons were lysed with 1% CHAPSO buffer (50 mM HEPES [Invitrogen], pH 7.4% and 1% CHAPSO [Sigma-Aldrich, St. Louis MO]) supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Pittsburgh PA); for the neurons pretreated with KCl, 100 mM CaCl₂ was added to the lysis buffer. The lysates were passed through a 27-gauge needle and were rotated for 1 hour at 4 °C. The protein concentrations were determined from supernatants using the Thermo Scientific Pierce BCA protein assay. Equal protein aliquots were incubated with either rabbit anti-GFP (Abcam, Cambridge, MA) as a control or rabbit anti-PS1 C-terminus (CT) (Cell Signaling, Danvers MA) antibodies on a rotator overnight at 4 °C. 50 μL of Protein G Dynabeads (Invitrogen) was added to each sample and incubated for 10 minutes at room temperature on a rotator. The beads were collected from the sample and washed twice with 1% CHAPSO buffer with appropriate supplements. The bound proteins were eluted from the beads using 1% CHAPSO buffer, 4X LDS sample buffer [Invitrogen], and 10% reducing agent [Invitrogen] and boiled at 95 °C for 5 minutes.

Western blotting was performed using NuPage 4–12% Bis-Tris Gel 1.5 mm (Invitrogen) in 1X MOPS running buffer (Invitrogen). Protein was transferred onto a nitrocellulose membrane with 0.45 micron pores that was then washed in TBST and incubated in Odyssey Blocking Buffer for 1 hour (LI-COR, Lincoln, NE). The membrane was probed with the following antibodies overnight at 4 °C on a shaker: mouse anti-Syt-1 (Millipore) or rabbit-anti-PS1 C-terminus (CT) (Cell Signaling). Respective HRP-conjugated secondary antibodies were applied for 1 hour at room temperature. Membranes were developed using ECL-Plus illuminating solution (Perkin Elmer Inc., Waltham, MA). The strength of the PS1/Syt-1 binding was estimated by measuring the optical density of the bands corresponding to pulled down Syt-1 and respective PS1 bands using ImageJ. This ratio was then normalized to the intensities of the input Syt-1 band.

2.4. Immunocytochemistry/immunohistochemistry

Primary neurons were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 detergent in 1.5% normal donkey serum (Jackson ImmunolResearch Laboratories, West Grove, PA) in phosphate-buffered saline. To assess PS1/Syt-1 proximity, cells were incubated overnight at 4 °C with rabbit anti-Syt-1 (Abcam) and mouse anti-PS1 (CT) (Sigma-Aldrich) antibodies. Respective Alexa Fluor 488 (A488) or Cy3-labeled secondary antibodies (Thermo Fisher Scientific, USA) were applied to the cells the following day for 1 hour light-protected at room temperature. Glass coverslips were applied with VectaShield mounting medium (Vector Laboratories Inc., Burlingame, CA). Fixed free-floating sections of mouse brain tissue were permeabilized using 0.4% Triton X-100 detergent in 1.5% or 3% normal donkey serum (Jackson ImmunolResearch Laboratories). Tissue was incubated overnight at 4 °C with goat anti-PS1 N-terminus (NT) (Millipore) and rabbit anti-Syt-1 (Abcam) antibodies, followed by a light-protected incubation with corresponding A488- or Cy3-labeled secondary antibodies for 1 hour. Tissue was mounted on Superfrost Plus Microscope slides (Fisher), and glass coverslips were applied using VectaShield mounting medium (Vector Laboratories Inc.).

2.5. Fluorescence lifetime imaging microscopy

The extent of the interaction between PS1 and Syt-1 was determined by measuring their relative proximity using FLIM, a
Förster resonance energy transfer (FRET)-based technique. Briefly, neurons and mouse tissue were immunostained with antibodies recognizing PS1 and Syt-1 and labeled with A488 acting as the donor, and Cy3 as the acceptor fluorophore, respectively. A Chameleon Ti:Sapphire laser (Coherent Inc., Santa Clara, CA) was used for two photon excitation at 780 nm and A488 lifetime was recorded using a high-speed photomultiplier tube (MCP R3809; Hamamatsu, Iwata City, Japan) and a fast-time correlated single-photon counting acquisition board (SPC 830; Becker and Hickl, Berlin, Germany). The lifetime of the donor fluorophore in the absence of an acceptor fluorophore was measured to establish a no-FRET A488 baseline (t1). In the presence of an acceptor, the donor fluorophore, when excited, produces reduced emission energy but only if the donor and acceptor are less than 5–10 nm distance apart. Thus, the donor lifetime is reduced (t2) and the degree of the donor lifetime shortening corresponds to the proximity between the donor and acceptor fluorophore-labeled proteins. All FLIM data acquired were analyzed using SPCImage software (Becker and Hickl), and FRET efficiency (%EFRET) was calculated using the following equation: \( \%EFRET = 100 \times (t1 - t2)/t1 \), where t1 and t2 are donor lifetimes in the absence and presence of an acceptor, respectively.

### 2.6. Calcium imaging

To measure baseline levels of intracellular calcium, primary neurons were transduced with adenovirus carrying AAV8-CBA-YC3.6 plasmids (generous gift from Dr. E. Hudry, Massachusetts General Hospital, Boston, MA), encoding a FRET-based calcium indicator—Yellow Cameleon—as described previously (Nagai et al., 2004). This ratiometric probe can be used to quantify the concentration of intracellular calcium by measuring the ratio of yellow fluorescent protein to cyan fluorescent protein fluorescence intensity. The construct was expressed in cells for 48–72 hours and the culture medium was replaced with Hank's Balanced Salt Solution with calcium and magnesium (Thermo Fisher Scientific, Waltham, MA) for imaging on the Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany). A Chameleon Ti:Sapphire laser (Coherent Inc.) excited the fluorophores at 860 nm, and the emitted fluorescence was split into seven channels between 456 and 617 nm using the Metadetector (cyan fluorescent protein emission peaked at 489 nm ± 10.7 nm, yellow fluorescent protein peaked at 531 nm ± 10.7 nm). Mean fluorescence intensity for each fluorophore was quantified using ImageJ software after background subtraction.

Alternatively, to measure the change in intracellular calcium levels after KCl stimulation, primary neurons were preloaded with 4 μM Oregon Green 488 BAPTA-1, AM (OGB) (Thermo Fisher Scientific) with Pluronic F-127 for 30 minutes at 37 °C. The medium was replaced with Hank's Balanced Salt Solution, containing calcium and magnesium (Thermo Fisher Scientific) for imaging on the Zeiss LSM 510. Using time-lapse settings, images were taken every 5 seconds starting 25 seconds before and ending 200 seconds after the 50 mM KCl solution was added. Previous studies have found the treatment with KCl invokes a change in the concentration of calcium that is within the linear range for the OGB indicator (Barish, 1991; Maravall et al., 2000; Paredes et al., 2008). For neurons in vitro, to quantify the change in intracellular calcium, the mean fluorescence intensity was quantified using ImageJ software after background subtraction, and the difference between the post-stimulation and prestimulation fluorescence intensities was normalized to the prestimulation fluorescence intensity as described in Zolotowska et al., 2016. The maximum change in fluorescence for the OGB calcium indicator was 30%–40%.

### 2.7. Statistics

Statistical analysis of the FRET efficiency, %EFRET, data was performed using GraphPad Prism 5 or Microsoft Excel software. Data were compared using a two-sided Mann-Whitney t-test in addition to the D’Agostino and Pearson omnibus normality test to evaluate the data distribution. ImageJ software was used to analyze band density of Western blot data. The band density between samples was compared in GraphPad Prism 5 using a one-way ANOVA with a Bonferroni correction. Values were considered significant at \( p < 0.05 \).

### 3. Results and discussion

We have previously shown that the number of Syt-1—positive puncta and the interaction between remaining Syt-1 and PS1/γ-secretase are significantly reduced in the cortex from sporadic AD cases with neuropathologically confirmed Braak stages V–VI (Zolotowska et al., 2017). The association between PS1 and Syt-1 in neurons is triggered by calcium (Kuzuya et al., 2016), the homeostasis of which is well known to be dysregulated in AD and aging (Kuchibhotla et al., 2008; Sharma et al., 2016; Thibault et al., 1998). However, the effect of normal aging on the PS1/Syt-1 interaction and the influence of age on the calcium dependence of PS1/Syt-1 binding remain unknown.

To address these questions, first, we cultured primary neurons in vitro to either DIV 8–14 (referred to as young) or DIV 21–28 (referred to as old). The cells were subsequently treated with the vehicle control or 50 mM KCl for 15 minutes to induce calcium influx, and thus PS1/Syt-1 complex formation. Using coimmunoprecipitation (co-IP), Syt-1 was pulled down from the neuronal lysate using anti-PS1 antibody, and levels of PS1 (IP efficiency) and the coinmunioprecipitated Syt-1 were measured via Western blotting. The ratio of Syt-1 to PS1 band intensities were then normalized to Syt-1 input band intensity to quantify PS1/Syt-1 complex formation. The interaction between PS1 and Syt-1 was elevated 1.79-fold in old neurons in comparison to young neurons at the baseline condition (−KCl) (Fig. 1A). This age effect also holds in the KCl-treated condition (+KCl), with KCl treatment increasing the PS1/Syt-1 association in both older and younger neurons. Although the analysis did not reach statistical significance, there was a consistent trend toward the stronger association of the two proteins with age, which led us to further investigate this phenomenon separately in cell bodies and processes of intact primary neurons using a complementary FRET-based assay, FLIM.

Based on the FLIM assay, there was a significantly increased interaction between PS1 and Syt-1 in both cell bodies and processes of the older neurons at the baseline conditions (Fig. 1B, −KCl). This effect may be related to differences in calcium regulation in young compared with old neurons. Previous studies have found that older neurons have decreased electrical excitability (Scott et al., 1988). In addition, older CA1 hippocampal neurons have increased levels of basal calcium and take longer to equilibrate intracellular calcium after calcium influx (Raza et al., 2007). These factors can lead to calcium dyshomeostasis and in turn affect proteins and protein interactions that respond to calcium. There are several mechanisms by which calcium homeostasis becomes dysregulated. One of these is the increased presence of reactive oxygen species in aging neurons, which affects ion channel—including voltage-gated calcium channel—functioning (Gorlach et al., 2015; Patel and Setzi, 2016; Perlodzi et al., 2016). Because the PS1/Syt-1 interaction is induced by calcium (Kuzuya et al., 2016), the observed increased interaction of PS1/Syt-1 in older neurons may be a result of differences in the baseline calcium levels in old neurons compared with young neurons. To test this in our experimental setup, a YC3.6 FRET-based
between 8-14 DIV (young) and 21-28 DIV (old) neurons. Mean processes of intact neurons. Neurons were treated with 50 mM KCl or vehicle for 15 minutes beforehand and corresponding antibodies. Mean normalized to band densities of young neurons that were not treated with KCl. Bars show mean ± SEM. N = 5-55 (ii) OGB was used to visualize differences in calcium influx in response to KCl treatment between 8-14 DIV (young) and 21-28 DIV (old) neurons. Mean ± SEM. N = 955-1051. ***p < 0.0001. Abbreviations: PS1, presenilin 1; Syt-1, synaptotagmin 1; DIV, days in vitro; FLIM, fluorescence lifetime imaging microscopy; OGB, Oregon Green 488 BAPTA-1, AM; FRET, Förster resonance energy transfer.

To further investigate how age would affect the calcium-triggered interaction between PS1 and Syt-1, we treated young and old neurons with KCl. In cell bodies, the KCl treatment did not lead to any significant differences in PS1/Syt-1 binding in either young or old neurons (Fig. 1B, baseline compared with +KCl). However, treatment with KCl still yielded a significant increase in the association of PS1 and Syt-1 in old compared with young neurons. In neuronal processes, however, the KCl treatment increased PS1/Syt-1 interaction in both young and old neurons by 1.85-fold and 1.5-fold, respectively, compared with the baseline (–KCl). The difference between cell bodies and processes in response to KCl may be explained by the fact that as neurons age, most functionally active Syt-1 translocate to the synapses. Furthermore, the OGB calcium indicator confirmed that there is a greater influx of calcium and/or higher intracellular calcium levels in older neurons compared with younger neurons in response to this same KCl treatment (Fig. 1C-ii). The fact that detected differences in the PS1/Syt-1 interactions between young neurons were statistically significant in the FLIM assay and not in the coinmunoprecipitation from the total cell lysates indicates that FLIM is more sensitive and better suited to detect changes in the protein association than coinmunoprecipitation, especially if the protein-protein interactions differ in distinct subcellular compartments, such as cell bodies and processes.

This increased influx in calcium may therefore be at least partially responsible for the increased association between PS1 and Syt-1 in older neurons compared with younger neurons when treated with KCl. This is in agreement with the finding that aging neurons experience an enhanced overshoot during firing (Allan et al., 2013; Scott et al., 1988), perhaps as a result of changes in ion channels during aging, including oxidation of these channels (Patel and Setsi, 2016). It has been reported that areas of the brain with increased neuronal activity, and thereby more calcium influx in neurons, are the areas of the brain where neurodegeneration typically commences in AD and where Aβ accumulates first (Braak et al., 2006; Sperling et al., 2009). The increased interaction between PS1 and Syt-1 in response to calcium influx could be a protective measure against neurodegeneration. We have previously reported that blocking the interaction between PS1 and Syt-1 alters synaptic vesicle cycling, results in PS1 adapting its pathogenic conformation, and increases the Aβ 42/40 ratio (Zolotowska et al., 2017). It is therefore likely that the increased interaction between PS1 and Syt-1 during aging is intended to protect the neurons from the effects of PS1 in its pathogenic conformation, which is increasingly found during aging (Wahlster et al., 2013). The increase in the interaction between PS1 and Syt-1 may be a way to remedy the tendency of PS1 to adapt its pathogenic conformation during aging. It is possible that this effect is an early event that later on is overwhelmed by chronic impairments eventually resulting in a decreased interaction between PS1 and Syt-1. Future longitudinal studies should investigate how sustained increases in calcium influx and/or chronic inflammation affect the association of the two proteins.

To determine if the PS1/Syt-1 association profiles in neurons in vitro are physiologically relevant, we analyzed the interaction between PS1 and Syt-1 in the hippocampal CA1 area, an area that is intimately involved in memory formation and is affected in AD. The FLIM assay was used to determine the effect of age on PS1/Syt-1 interaction in vivo in young (5 months old) and old...
(23–24 months old) wild-type mice. We found that there was a strong effect of age on the interaction, with significantly higher % E_FRET in old mice compared with that in younger mice in both neuronal cell bodies and processes (Fig. 2). Of note, this effect was stronger in processes (p < 0.0001) than in cell bodies (p = 0.015), presumably because the higher levels of mature functionally active Syt-1 in processes accounts for preferential, closer interactions with PS1.

This study reports increased interaction between the AD-related protein PS1 and the synaptic vesicle protein, Syt-1, in mouse brain during normal aging and in neurons “aging” in vitro. We show that older cultured neurons have significantly higher baseline levels of calcium and display increased influx of calcium in response to treatment with KCl, effects that possibly augment the calcium-mediated PS1/Syt-1 complex formation. The experiments in this study report a rather acute effect of calcium overload after 15 minutes of KCl treatment on the PS1/Syt-1 interaction. It remains unknown what the long-term effects of increased calcium influx—and/or chronically elevated neuronal activity—may be on these two proteins in vivo. Given the diminished PS1/Syt-1 interaction in sporadic AD brains, neuropathological changes in the brain may decrease association of the two proteins over time. Eliciting changes in neurons during normal aging can not only help understand the mechanism of normal aging but also to gain insight into how neurodegenerative processes differ from aging, and potentially highlight new pathways for future drug targets in the brain. Based on the interaction profile of PS1 and Syt-1 in AD and normal aging and the protective effects of this interaction (Zoltowska et al., 2017), future studies may reveal how to target this interaction to mitigate the effects of neurodegeneration.

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors’ contributions: NS and LK performed experiments, analyzed data, and drafted the manuscript. SS designed the study, performed experiments, and analyzed data. KZ, MM, and OB reviewed data and revised the manuscript. All authors read and approved the final manuscript.

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