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Control of neuronal ryanodine receptor-mediated calcium signaling by calsenilin

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Abstract

Calsenilin is a calcium ion (Ca^{2+}) binding protein involved in regulating the intracellular concentration of Ca^{2+} , a second messenger that controls multiple cellular signaling pathways. The ryanodine receptor (RyR) amplifies Ca^{2+} signals entering the cytoplasm by releasing Ca^{2+} from endoplasmic reticulum (ER) stores, a process termed calcium-induced calcium release (CICR). Here, we describe a novel mechanism, in which calsenilin controls the activity of neuronal RyRs. We show calsenilin colocalized with RyR2 and 3 in the ER of mouse hippocampal and cortical neurons using immunocytochemistry. The underlying protein-protein interaction between calsenilin and the RyR was determined in mouse central nervous system (CNS) neurons using immuno-precipitation studies. The functional relevance of this interaction was assayed with single channel electrophysiology. At low physiological Ca^{2+} concentrations, calsenilin binding to the cytoplasmic face of neuronal RyRs decreased the RyR's open probability, while calsenilin increased the open probability at high physiological Ca^{2+} concentrations. This novel molecular mechanism was studied further at the cellular level, where faster release kinetics of caffeine-induced Ca^{2+} release were measured in SH-SY5Y neuroblastoma cells overexpressing calsenilin. The interaction between calsenilin and neuronal RyRs reveals a new regulatory mechanism and possibly a novel pharmacological target for the control of Ca^{2+} release from intracellular stores.

Keywords

Alzheimer's disease; calcium; calsenilin; cortex; hippocampus; ryanodine receptor; RyR

Introduction

Calcium plays an important role in numerous cell signaling pathways in neuronal cells, some of which include memory consolidation, vesicle release, and apoptosis. Due to the ubiquitous nature of intracellular calcium, vital key pathways are employed to convert subcellularly localized alterations in calcium concentrations into global cellular responses.¹ Propagation of intracellular calcium signals throughout the cell is accomplished through

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calcium release channels such as ryanodine receptors (RyRs) located on intracellular calcium sources, most notably the endoplasmic reticulum.² Calcium binding proteins communicate changes in calcium levels through conformational changes that effect phosphorylation, protein binding, and the action of target proteins.³ The neuronal calcium sensor family of proteins provide high affinity calcium-binding sites that alter neuronal cell signaling affecting a diverse range of processes, such as memory formation, vesicle release, and neurite outgrowth.⁴ In addition, a range of proteins mediate uptake of Ca^{2+} into intracellular stores and extrusion of Ca^{2+} into the extracellular space, which shapes cytoplasmic calcium signaling. These two intimately connected processes, calcium release and calcium sequestration, work synergistically to shape calcium signaling throughout the cell.

RyRs are major calcium- induced calcium release (CICR) channels located on the endoplasmic reticulum membrane in neurons and most other cell types.⁵ RyRs are found throughout the nervous system^{2,6,7} and control multiple processes in neurons through CICR including vesicle release, membrane depolarization, and apoptosis.¹ RyRs contain a large N-terminal cytoplasmic domain, which interacts with proteins, small molecules, and signaling molecules modulating CICR mediated by this channel.^{3,8} Of the three RyRs, RyR1 was originally described in skeletal muscle, RyR2 in heart tissue, and RyR3 in brain tissue, while all three are differentially expressed in most cell types and tissues.¹⁻³ The three types of RyRs differ in their dependence on the cytosolic Ca^{2+} concentration for activation and inhibition, which combined with their differential distribution, results in tissue-specific calcium release producing cell- and tissue-specific functions.² Previous studies have identified protein-protein interactions between RyRs and cytoplasmic proteins resulting in a change in RyR ion channel activity and Ca^{2+} release from the ER.^{9,10} At the same time, in both *in vitro* and *in vivo* disease models of Alzheimer's disease (AD), increased release of Ca^{2+} from ryanodine-sensitive stores was observed in diseased cells,¹¹⁻¹⁴ leading to increased apoptosis of neuronal cells^{15, 16}. Similarly, inhibitory control of RyR activity reduced cell damage and cell death in *in vitro* models of Parkinson's disease⁵⁷ and multiple sclerosis⁵⁸ and in *in vivo* models of neuroinflammation⁵⁹ and Huntington's disease⁶⁰ resulting in reduced functional impairment⁵⁹⁻⁶⁰.

Here, we investigated the interactions between calsenilin, a calcium-binding protein also known as KChIP3/DREAM, and RyRs, specifically RyR2 and 3 and determined the impact of this interaction on calcium signaling. Calsenilin is a 31kDa protein of the neuronal Ca^{2+} sensor family¹⁷ with four C-terminus EF-hand motifs and a unique N-terminus that is responsible for binding to target molecules¹⁸⁻²⁰. Calsenilin is found in the human brain²¹ and is expressed throughout the CNS, with particularly high expression levels in layers V and VI of the cerebral cortex as well as in the granule layers of the cerebellum, olfactory centers and the hippocampus,²²⁻²⁶ and it is involved in synaptic plasticity.²⁷⁻²⁹ Calsenilin has also been identified in dorsal root ganglia lacking Kv4.3 expression,³⁰ $\text{A}\beta$ mechanoreceptors, $\text{A}\sigma$ sensory receptors and some C-fiber peptidergic nociceptors³⁰ of the spinal cord.

Calsenilin interacts with the C-terminus of presenilin 1 and presenilin 2 to stabilize the proteolytic fragments of both presenilin 1 and presenilin 2¹⁹ and enhance the enzyme

activity of the presenilin- γ -secretase complex.³¹ Calsenilin alters cellular Ca^{2+} dynamics through several mechanisms, such as controlling the gating kinetics of $\text{Kv}_{4.2}$ potassium channels,¹⁸ interacting with the downstream regulatory element (DRE) sequence and modulating the transcription of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger NCX3,^{20, 32} reversing presenilin 1 mediated enhancement of Ca^{2+} release,³³ altering the Ca^{2+} concentration of endoplasmic reticulum stores,^{32, 34, 35} and increasing Ca^{2+} induced apoptosis in both cellular and mouse models of AD.^{36, 37}

Here, we propose that calsenilin acts as a regulator of RyR-mediated CICR that could be used as a potential drug target for controlling calcium dysregulation in neurodegenerative diseases such as AD. Our study shows that calsenilin and brain RyRs, neuronal RyR2 and RyR3, co-localize and interact directly, which results in altered RyR-mediated Ca^{2+} release at the single channel level. Our functional data from electrophysiology and calcium imaging experiments show that calsenilin alters the Ca^{2+} release kinetics of RyRs at the single-channel level in isolated microsomes, and of RyR-channel populations in neuroblastoma cells overexpressing calsenilin. Taken together, these results suggest a novel mechanism whereby calsenilin controls RyR2- and RyR3-mediated CICR release in neurons through direct protein-protein interaction.

Materials and Methods

Immunocytochemistry

NeuroPure embryonic day 18 (E18) Sprague Dawley rat cortical cells were prepared following manufacturer's protocol (cat. #N200200, Genlantis, San Diego, CA). Immunostaining was conducted as described previously.^{38, 39} Briefly, media was removed and attached cells were fixed for 20 min in 4% PFA (0.01M PBS, pH 7.4) at room temperature (RT). The fixative was removed, followed by three 10 min washes using PBS (0.01 M PBS, pH 7.4). Blocking solution (10% normal donkey serum, 1% BSA, and 0.05% Triton-X 100 in 0.01 M PBS) was added and cells were incubated for 1 hour at RT. After blocking, the cells were incubated with the primary antibodies: DREAM (1:50; sc-9309, Santa Cruz Biotechnologies, Dallas, TX), RyR2 (1:10,000; AB9080, Chemicon Biotechnologies, Temecula, CA), RyR3 (AB9082 (1:5,000; Chemicon Biotechnologies) in incubation buffer (3% normal donkey serum, 1% BSA and 0.05% Triton-X 100 in 0.01 M PBS) overnight at 4 °C. Following primary incubation cells were washed three times for 10 minutes with PBS. Cells were next incubated with secondary antibodies: AlexaFluor 488 (1:2,000; A11055, Life Technologies, Carlsbad, CA) and AlexaFluor 594 (1:2,000; A11062, Life Technologies), and DAPI (1:20,000) for 1 hour at RT. After three washes, coverslips were mounted on slides using Aqua-Polymount (Polysciences, Warrington, PA). Negative controls consisted of the omission of the primary antibody.

Preparation of Mouse Brain Sections

C57BL/6 mice (Jackson Laboratories, Bar Harbor, MA) were euthanized using the IACUC approved method of carbon dioxide overdose. Brains were removed from the cranial cavity and immersion fixed in 4% PFA at 4 °C overnight. The tissue was then sequentially cryoprotected in a graded series of 10%, 20%, and 30% sucrose at 4 °C in PBS. Tissue was

removed from final sucrose solution and placed in Peel-A-Way® mold (R-30, Polysciences Inc., Warrington, PA), and incubated at the Tissue-Tek optimal cutting temperature (OCT) embedding medium (Tissue Tek, Torrance CA) for 1hr at 4 °C. The samples were then placed in –80 °C isopentane (Alfa Aesar, Ward Hill, MA) to solidify the OCT compound and snap-freeze the tissue. Molds were sectioned at 30 µm using a Leica model CM3050 S cryostat.

Immunohistochemistry

Immunohistochemistry was performed as stated previously in the immunocytochemistry section with the addition of the following steps. Before blocking, tissue was placed in 1mg/ml sodium borohydride (Sigma Aldrich) three times for 10 minutes.⁴⁰ After secondary antibody incubation and subsequent washes brain sections were incubated in 25mM CuSO₄ for 10 minutes. The sections were subsequently washed three times with PBS for 10 minutes, followed by a 10-min wash in deionized water. Coverslips were applied using Aqua-Polymount.

Confocal Microscopy

All immunostaining and colocalization images were acquired on a Leica SP5 white-light laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) with a 63x glycerin-immersion objective. Alexa 488 was excited at 490 nm and collected at 505–550 nm. Alexa 594 was excited at 594 nm and collected at 599–650 nm. Immunocytochemistry optical sections (z-stack with 0.13 µm z-step, 1.6 optical slice thickness) and immunohistochemistry optical sections (z-stack with 0.13 µm z-step 1.0 optical slice thickness) were collected (2048 x 2048 pixel images) running Leica Application Suite Advanced Fluorescence v 2.6. To ensure uniformity of labeling, all images were acquired with same the confocal settings (pinhole size, gain, offset, and laser power). To decrease signal bleed through, images were collected in the order of Alexa 488, Alexa 594, and DAPI.

Statistical Analysis of Colocalization

Colocalization and intensity analysis were conducted with MBF ImageJ (McMaster Biophotonics Facility, Hamilton, ON) utilizing the Pearson's and Mander's Coefficient.⁴¹ The Pearson's coefficient and Mander's coefficients were validated using the Costes method plugin for Fiji ImageJ.

Recombinant Protein

Glutathione S-transferase (GST) tagged calsenilin protein was made by transforming bacteria, strain BL21(DE3) pLysS (Novagen, Madison, WI) with a cDNA construct of the full length mouse calsenilin, as described previously.⁹ Briefly, calsenilin protein expression was induced by 100 µM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 28 °C for 4 h. Bacterial cultures were then pelleted, lysed, and centrifuged at 18,000×g at 4 °C for 20 min. Bacterial protein was extracted by incubation in 50 µl of immobilized GST resin (Novagen, Madison, WI) at 4 °C for 2 h, and washed in PBS. Recombinant protein was eluted with a buffer containing 10 mM reduced glutathione.

Co-immunoprecipitation

Co-immunoprecipitation and Western blotting was conducted as described previously.^{9, 39} Briefly, ER microsomes (250 µg protein per sample) were prepared from adult mouse whole brains as described previously.^{9, 10, 42} Samples were incubated alone, with RyR2 antibody (AB9080), or anti-rabbit IgG (2 mg/ml; Jackson Laboratories, Baltimore, PA) with or without recombinant calsenilin protein with a GST tag (combined molecular weight was between 51–55 kDa) under constant shaking at 4 °C for 12 hrs. Following incubation, samples were combined with protein A magnetic beads (EMD Millipore) for 10 minutes at RT, washed three times in PBS with 0.1% Tween 20, and eluted with Western Blot Sample Buffer (10%, glycerol: 10%, β-mercaptoethanol: 1%, bromophenol blue: 0.004%, tris (hydroxymethyl) aminomethane (Tris)-HCl: 0.5 M, pH 6.8) at 80 °C for 10 min.

Western Blotting

Solubilized proteins and molecular weight markers (HiMark Pre-stained Protein Standard, Thermo Fisher Scientific, Waltham, MA) were separated on a 3–8% NuPAGE® Novex® Midi Tris-acetate gel (Life Technologies) and transferred to Amersham Hybond-ECL blotting paper (0.45µm, GE Healthcare). Blots were incubated for 12–16 hrs at 4 °C with the calsenilin antibody (05-756) (1:500; Upstate, Lake Placid) and a RyR2 antibody (MA3–916) (1:100; Thermo Fisher Scientific). Blots were incubated with a horseradish peroxidase (HRP)-linked secondary antibody (31430) (1:5000; Thermo Fisher Scientific) at RT for 1 hour. Membranes were developed using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged on the Syngene G: BOX using Syngene GeneSnap acquisition software.

Single Channel Electrophysiology

Electrophysiology was conducted as previously stated [17, 18, 32]. Microsomes were added to the *cis* compartment of the bilayer chamber (Warner Instruments, Hamden, CT), where they were fused to a lipid bilayer formed with a 3:1 mixture of dried phosphatidylethanolamine/phosphatidylserine lipids (Avanti Polar Lipids, Alabaster, AL) on a 150 µm diameter aperture using hyperosmotic conditions (KCl solution of ~300–500 mM). The microsome incorporation was monitored by the appearance of channel activity and followed by perfusion of the *cis* chambers with 10 volumes of *cis* (106 mM Tris–OH, 220 mM HEPES, pH 7.35, ~320 mOsm) buffer. RyR mediated single channel currents were activated by addition of Ca²⁺ into the *cis* chamber at fixed levels with rationed amounts of CaCl₂/EGTA calculated using MaxChelator software (Stanford University). Recombinant calsenilin protein was added to the *cis* side of the chamber. The RyR blocker Ruthenium Red (10–25 µM) was routinely added at the end of the experiment to verify the identity of the RyRs. Experiments were performed at RT with single-channel currents recorded at a holding potential of 0mV (*trans* chamber grounded) using a BC-525 amplifier (Warner Instruments), filtered at 500 Hz and digitized at 5 kHz using pCLAMP™10 software (Molecular Devices, Sunnyvale, CA). pCLAMP™10 was used to conduct offline current trace filtering (320 Hz), to determine dwell time, to provide an amplitude histogram and to calculate open-channel probability calculations.

Single-Channel Electrophysiology Statistics

All recordings were normalized to control values and reported as percentage of controls. GraphPad Prism 5 (*GraphPad Software, Inc.*, San Diego, CA) was used for all analysis and data presentation. Nonlinear curve fitting was performed using the Levenberg–Marquardt algorithm and tested for goodness of fit using the chi squared algorithm. A one-way ANOVA with a Dunnett's post hoc test was used to test significance (Mean \pm SEM; $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Transfection

Calsenilin cDNA was prepared from total RNA using SuperScript™ (Invitrogen) II Reverse Transcriptase (RT) (Thermo Fisher Scientific) and poly T primers, as described previously.⁹ SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA) were co-transfected per manufacturer's instruction using a Lonza 4D-nucleofector transfection unit (Lonza, Walkersville, MD). Briefly, cells were suspended in Lonza transfection media with 2 μ g of calsenilin pcDNA3.1 (+) Zeo mammalian expression vector and 0.5 μ g of tdTomato (reporter protein) pcDNA3.1 (+) Zeo mammalian expression vector. Transfected cells were then plated at 25,000 cells per laminin/poly-d lysine coverslips (BD Biocoat, Bedford, MA) using SH-SY5Y media (10% fetal bovine serum (PAA, Piscataway, NJ), 1:200 penicillin/streptomycin, 50% Ham's F-12 media, 50% eagle minimum essential media (Lonza, Walkersville, MD) and maintained at 37 °C, 5% CO₂, 95% O₂ for 36–48 hours.

Calcium Imaging

Media from mock- or calsenilin-transfected SH-SY5Y cells were removed and washed in extracellular solution buffer (ECS) (137 mM NaCl, 5mM KCl, 1 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM HEPES, 22 mM D-(+)-glucose, and 1.8 mM CaCl₂; pH 7.35 at 37 °C); then, cells were incubated in 2 μ M fura-2 AM (Invitrogen) in ECS for 30 minutes. Coverslips were washed with ECS containing 55 mM KCl to empty and subsequently refill their intracellular Ca²⁺ stores; they were then washed two more times with ECS. Coverslips were assembled to the imaging platform and continually perfused at 2 ml/minute with ECS at a constant temperature of 37 °C. Perfusion protocols were the same for all experiments and controlled through MetaFluor sequential journals. The perfusion protocol was as follows: 1 minute of ECS to establish a baseline, 3 minutes of 30 mM caffeine in ECS to stimulate the RyRs, and 3 minutes of ECS to return the response to baseline. Regions of Interest (ROIs) were selected following Ca²⁺ imaging to adjust for drift. Background ROI's (N = 4 for each coverslip) were selected using a region of the optical field not occupied by cells.

Calcium Imaging Statistical Analysis

GraphPad Prism 5 was used for all analysis and data presentation. The calculation for the area under the curve (AUC) was defined as follows: the beginning of the curve was the time point where the Ca²⁺ value rose above 5% of baseline, and the ending of the curve was defined by the last point in the curve that was above 5% of the baseline. Both maximum value and width of response were calculated from this curve. Linear regression was done to calculate the slope values of each response using the max value and the first point 5% above

the baseline. All values were averaged and statistically analyzed using the student's t-test (Mean \pm SEM, $p < 0.05$, * $p < 0.01$, ** $p < 0.001$ ***).

Results

Calsenilin and RyR are co-localized and have a direct protein-protein interaction in neuronal tissue

Figure 1 shows typical calsenilin and RyR2 or RyR3 (Figure 1A) staining patterns in primary cultured cortical neurons and RyR2 in the dentate gyrus and cortical layer VI of a 6-week-old C57 mouse brain (Figure 1B). Co-localization between the two proteins is highest in the perinuclear region of the cells for both RyR2 and RyR3. This staining pattern is indicative of ER Ca^{2+} release channels in neuronal tissue.^{43–45} Figure 1C shows this pattern of co-localization, which appears for RyR2 and calsenilin in all cortical neurons, hippocampal and cortical layers tested. Co-localization of RyR3 and calsenilin in both the hippocampus and the cortex is region-specific with higher co-localization seen in the CA3 region of the hippocampus and cortical layer VI (Figure 1C). Immunoblotting of proteins captured using an anti-RyR2 antibody (Fig 1D) shows expression of both RyR2 (approximately 550 kDa) and calsenilin recombinant protein with a GST tag (approximately 51 kDa). Substituting the anti-RyR2 antibody with IgG yielded no bands for RyR2 or calsenilin (Fig 1D), proving the specificity of the anti-RyR2 antibody. Together these data suggest that calsenilin and RyR2 /RyR3 not only reside in the same subcellular location, but interact.

Calsenilin Alters RyR Single Channel Ca^{2+} Release in Single Channel Electrophysiological Experiments

Figure 2 shows typical recordings of RyR activity at physiological intracellular calcium ion concentrations of 100 nM (pCa7; Figure 2A), 1 μM (pCa6; Figure 2C), and 10 μM (pCa5; Figure 2E) before and after calsenilin addition. The pCa7 amplitude histogram (Figure 2B) shows that calsenilin reduces the number of times the channel opens and decreases the duration of each opening. The decrease in the number and duration of channel openings translates to a significant decrease in the open probability of the subconductance state (2 pA) of the RyR (Figure 2G). This data suggests that at a resting cytosolic Ca^{2+} concentration, calsenilin decreases Ca^{2+} release from RyRs. Conversely, at higher Ca^{2+} concentrations, calsenilin has the opposite effect on RyR single-channel kinetics. After the addition of calsenilin (Figure 2C and 2D), RyR activity increases, characterized by an increase in the number of times the channel opens and the duration of each opening (Figure 2D). The changes in RyR activity after the addition of calsenilin at 1 μM Ca^{2+} translates to a significant increase in the open probability of the channel at the subconductance (2 pA; Figure 2G) and full conductance state (4 pA; Figure 2H). Similarly, the activity of the RyR at 10 μM Ca^{2+} after the addition of calsenilin shows increases in frequency and duration of openings (Figure 2E–H), translating into a significant increase of the full conductance state (4 pA; Figure 2H). At 100 μM Ca^{2+} , the addition of calsenilin did not result in significantly different RyR channel activity (data not shown). The data indicates that calsenilin affects the Ca^{2+} release kinetics of the RyR in a Ca^{2+} concentration dependent fashion. Calsenilin causes a sharpening of the CICR of RyRs by significantly decreasing the activity of the

receptor in a normally low-activity state (100 nM Ca²⁺) and increasing receptor activity at high activity states (1 μM and 10 μM, respectively).

Calsenilin Overexpression in SHSY-5Y Neuroblastoma Cells

Alters Caffeine-Induced Ca²⁺ Release—Figure 3 shows a typical caffeine-induced Ca²⁺ response in SHSY-5Y control cells (black line) and calsenilin overexpressing cells (red line). Calsenilin overexpression increases the release rate of Ca²⁺ from RyR sensitive stores compared to control cells. Calculation of the slope of Ca²⁺ release for both control and calsenilin overexpressing cells shows a significant increase in the rate of Ca²⁺ release (Figure 3B). The increased calcium release rate corresponds to a significant decrease in the duration of Ca²⁺ release of calsenilin overexpressing cells as compared to control cells calculated by the width of Ca²⁺ release (Fig 3D). The maximum amplitude of caffeine-induced Ca²⁺ release is significantly increased in calsenilin overexpressing cells compared to control cells (Fig 3C). Interestingly, a calculation of the area under the curve, which is a relative measure of the amount of Ca²⁺ released, shows no difference between control cells and calsenilin overexpressing cells (Fig 3E). Together these data suggest that calsenilin modulates CICR kinetics of the RyR.

Discussion

We have shown that calsenilin and RyR2 and RyR3 co-localize in the endoplasmic reticulum of mouse hippocampal and cortical neurons (Figure 1A–C), and have a probable protein-protein interaction between calsenilin and RyR2 in microsomes derived from brain tissue (Figure 1D). We found that calsenilin causes calcium ion concentration-dependent alterations in the Ca²⁺ release from single brain RyRs (Figure 2) and alters the kinetics of caffeine-induced calcium release from intracellular stores in a whole cell paradigm (Figure 3). The modulation of single channel RyRs by calsenilin decreased channel activity at lower 100-nM calcium concentrations (Figure 2A, B, G and H), and increased channel activity at 1 μM and 10 μM calcium concentrations (Figure 2C–F, G and H). In a whole cell paradigm, calsenilin modulation of multiple RyRs caused faster and higher amplitude caffeine-induced calcium release in SHSY-5Y cells overexpressing calsenilin (Figure 3). The alteration in calcium release dynamics from RyRs can have profound effects on the functioning of neurons.

Transient increases in calcium concentration in neurons can lead to opposite responses based on magnitude, timing, and duration of the elevated calcium release. For example, the consolidation, or lack of consolidation, of memories through long-term potentiation (LTP) and long-term depression (LTD) is a function of firing patterns in pre- and post-synaptic neurons.⁴⁶ The magnitude and timing of calcium signals in neurites decides whether a signal received by a post synaptic neuron will lead to LTP or LTD. LTP is a function of large rapid calcium transients, while LTD is a function of slow longer duration calcium transients.⁴⁷ These two phenomena are profoundly affected by release from intracellular calcium stores such as the RyRs and inositol triphosphate receptors (IP3Rs).⁴⁸ Further, neuronal calcium sensors that affect the magnitude of calcium signals in neurons can change the polarization of growth cones, branching of neurites, and rate of elongation.⁴⁷ The novel interaction

between calsenilin and RyRs describes a modulatory mechanism that can alter duration, timing, and magnitude of calcium transients in neurons.

Alterations in calcium-induced calcium signaling in neurons is implicated in several neurodegenerative diseases (Parkinson's disease⁵⁷; multiple sclerosis⁵⁸; neuroinflammation⁵⁹; Huntington's disease⁶⁰; reviewed in ⁴⁹). The long-standing calcium hypothesis of AD states that alterations in calcium homeostasis occur before the clinical symptoms of AD, but lead to the slow progressive decline of memory and cognition in sporadic AD (reviewed in ⁵⁰). Further, altered RyR calcium release in the dendrites of pre-symptomatic 3xTg-AD mice⁵¹ and young AD mice⁵² shows RyR-mediated release affects synaptic plasticity in hippocampal and cortical neurons. Calsenilin is expressed in hippocampal and cortical dendrites and is involved in synaptic plasticity.^{27–29} Mellström *et al.* found dendritic changes in the hippocampal neurons of a transgenic mouse model expressing the daDREAM mutant, suggesting calsenilin is involved in structural plasticity in the hippocampus.²⁸ In the present study, we have shown that calsenilin and RyR2 have a high degree of co-localization in proximal dendritic branch points (Fig 1B) indicating that this association occurs in more distal dendritic branches. Dysregulation of calcium homeostasis leads to an increase in resting calcium concentrations seen in both the soma⁵³ and dendrites⁵⁴ of neurons affected by AD, as well as in neurons of *in vivo* models of neuroinflammation⁵⁹ and Huntington's disease⁶⁰.

The association of calsenilin with RyRs increases calcium release at higher calcium levels and leads to faster and higher amplitude calcium transients in cells overexpressing calsenilin. The increase in calsenilin⁵⁵ and RyR⁵⁶ expression in AD, as well as increased resting calcium concentration in AD^{53–54}, *in vivo* models of neuroinflammation⁵⁹ and Huntington's disease⁶⁰ taken together with the increase in RyR activity in the presence of calsenilin and high calcium concentrations indicates a role for this interaction in the etiology of AD, and potentially other neurodegenerative diseases, such as Parkinson's disease⁵⁷, multiple sclerosis⁵⁸, neuroinflammation⁵⁹ and Huntington's disease⁶⁰.

From this data, we propose that calsenilin has an important modulatory role in calcium release from RyRs by which calsenilin acts as a filter on calcium-induced calcium release. By silencing the receptor at low physiological calcium concentrations and increasing the activity at higher physiological calcium concentrations, calsenilin sharpens calcium release from RyRs mimicking ion signals seen in neuronal cells. This novel interaction could be exploited pharmacologically to reduce calcium levels in neurodegenerative disorders by displacing calsenilin from the RyR. The exact binding sites and calcium dependence of this interaction must be explored to further utilize this potential tool for the alleviation of neuronal dysfunction in neurodegeneration.

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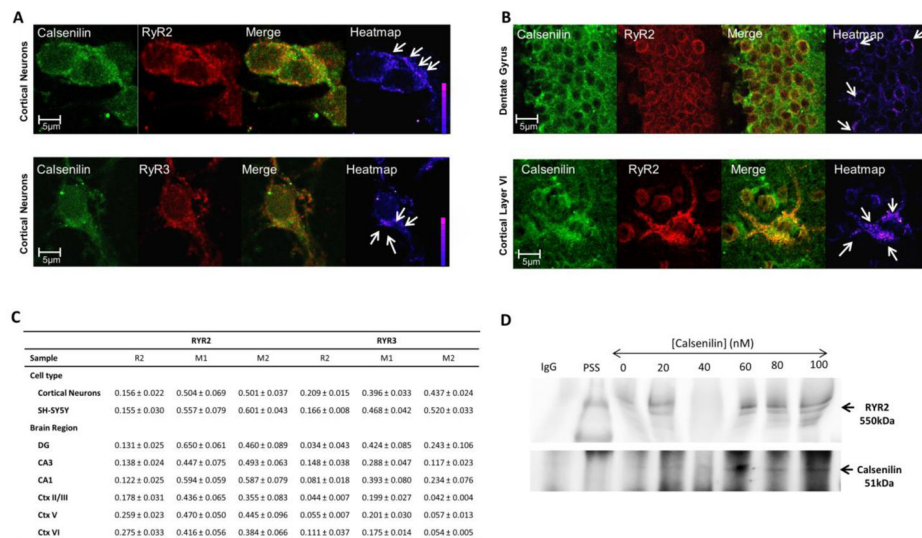


Figure 1. Calsenilin and RyR are co-localized and have a direct protein-protein interaction in neuronal tissue

Calsenilin (green) and A) RyR2 or RyR3 (red) immunoreactivity in E18 primary cultured cortical neurons B) RyR2 (red) expression in the dentate gyrus and cortical layer VI show a perinuclear staining pattern in regions adjacent to the nucleus indicative of ER staining (Merge) and a high degree of punctate co-localization in the perinuclear region (+PDM) as indicated by arrows and LUT in the last panel. C) Co-localization measurements between calsenilin and RyR2 or RyR3 for neuronal cell types (E18 primary cortical neurons and SH-SY5Y neuroblastoma cells) and 6-week-old C57BL/6 mouse brain sections (dentate gyrus, CA3, CA1, cortical layers II/III, V, VI). The R_r = Pearson's coefficient and M1 = Mander's coefficient describe the amount of calsenilin co-localized with the two RyR subtypes, whereas the M2 = Mander's coefficient describes the amount of RyR2 or RyR3 co-localized with calsenilin. Statistical significance represents the SEM for each replicate; co-localization was tested with the Costes method to ensure true co-localization. D) Western blot shows the presence of calsenilin in the brain-derived ER microsomes incubated with calsenilin protein and subjected to co-immunoprecipitation separation using the RyR2 antibody; the IgG control is shown in lane "IgG", and molecular weight standards are shown in lane "PSS". Abbreviations: RyR, ryanodine receptor; DG, Dentate Gyrus; CA1 or CA3, Cornu Ammonis 1 or 3; Ctx, Cortex layer; IgG, Immunoglobulin G; MW, molecular weight; PSS, pre-stained standard.

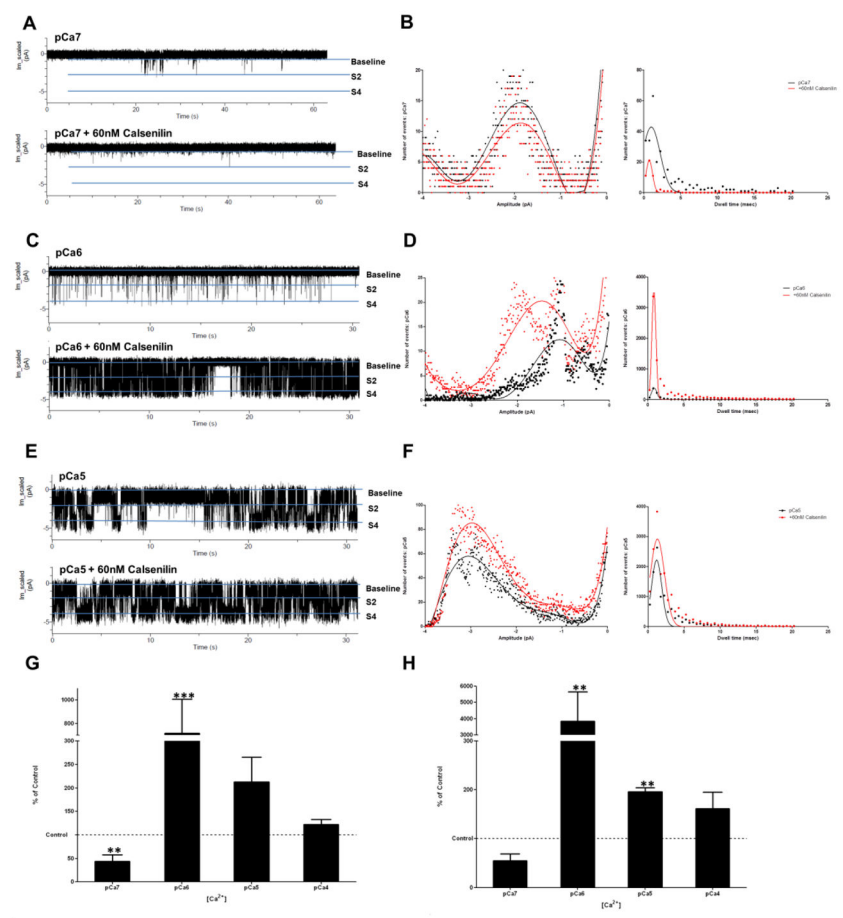


Figure 2. Calsenilin causes calcium concentration-dependent alterations in the biophysical Ca²⁺ release from single-brain RyRs

Representative planar lipid bilayer 60 sec traces (A, C and E) at varying physiological calcium concentrations in the presence (bottom trace) and absence (top trace) of calsenilin, and respective histograms (B, D and F) for amplitude (pA, left) and dwell time (msec, right). Graphs G and H show the open probability of the RyR in the presence of 60 nM calsenilin for $i_0 = -2$ pA and -4 pA, respectively, expressed as the percentage of the respective controls recorded in the absence of calsenilin at pCa4-7 (one-way ANOVA with Dunnett's post-test; values are represented as mean \pm SEM; ***, $p < 0.001$ and **, $p < 0.01$; $n = 3-8$).

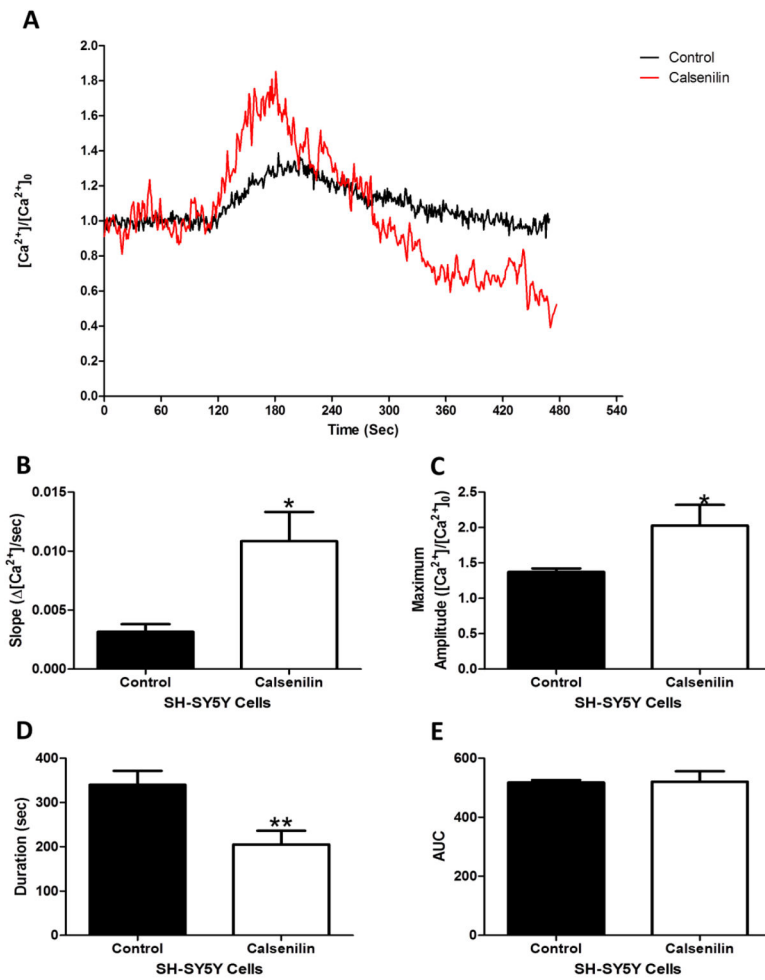


Figure 3. Calsenilin changed the kinetics of caffeine-induced calcium release

(A) Representative trace showing caffeine-induced calcium release from untransfected (black) and calsenilin-transfected (red) SH-SY5Y cells, and (B) the respective kinetic analysis of slope, (C) maximum amplitude, (D) duration, and (E) area under the curve (E). Significance parameters were obtained using Student's t-test. Values represented as Mean SEM * $p < 0.05$, ** $p < 0.01$, $n = 3-8$.