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Store depletion-induced *h*-channel plasticity rescues a channelopathy linked to Alzheimer's disease

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Abstract

Voltage-gated ion channels are critical for neuronal integration. Some of these channels, however, are mis-regulated in several neurological disorders, causing both gain- and loss-of-function channelopathies in neurons. Using several transgenic mouse models of Alzheimer's disease (AD), we find that sub-threshold voltage signals strongly influenced by hyperpolarization-activated,

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Appendix A. Supplementary material

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cyclic nucleotide-gated (HCN) channels progressively deteriorate over chronological aging in hippocampal CA1 pyramidal neurons. The degraded signaling via HCN channels in the transgenic mice is accompanied by an age-related global loss of their non-uniform dendritic expression. Both the aberrant signaling via HCN channels and their mislocalization could be restored using a variety of pharmacological agents that target the endoplasmic reticulum (ER). Our rescue of the HCN channelopathy helps provide molecular details into the favorable outcomes of ER-targeting drugs on the pathogenesis and synaptic/cognitive deficits in AD mouse models, and implies that they might have beneficial effects on neurological disorders linked to HCN channelopathies.

Keywords

HCN channel; Endoplasmic reticulum; TRIP8b; Carvedilol; Electron microscopy; Patch-clamp; Array tomography

1. Introduction

The age-related emergence of many forms of neuronal dysfunction is linked to dyshomeostasis of intracellular Ca^{2+} (Refs. (Khachaturian, 1984; Khachaturian, 1989; Gibson and Peterson, 1987; Landfield, 1983; Landfield, 1987). Indeed, studies have shown that inadequate Ca^{2+} regulation in aging and AD derives from altered gating of both external and internal Ca^{2+} sources (Arundine and Tymianski, 2003; Berridge, 2014; Bezprozvanny and Mattson, 2008; Chakroborty and Stutzmann, 2014; Del Prete, Checler, & Chami, 2014; LaFerla, 2002; Oh, Oliveira, & Disterhoft, 2010; Popugaeva and Bezprozvanny, 2013; Stutzmann and Mattson, 2011; Thibault, Gant, & Landfield, 2007). Interactions between these two sources are critical events that maintain cytosolic Ca^{2+} concentrations within a narrow range (Berridge, 1997), preventing sustained levels that can result in cell dysfunction and death (Choi, 1992; Disterhoft and Oh, 2006).

One form of cell dysfunction linked to overabundant free Ca^{2+} in the aged brain is exacerbated accommodation, driven by a larger Ca^{2+} -dependent afterhyperpolarization (AHP) following a burst of axonal action potentials (APs; (Disterhoft and Oh, 2006; Kaczorowski, Sametsky, Shah, Vassar, & Disterhoft, 2011; Kumar, Bodhinathan, & Foster, 2009; Landfield and Pitler, 1984; Moyer, Power, Thompson, & Disterhoft, 2000; Thibault and Landfield, 1996). Such high Ca^{2+} concentrations are a product (alone or in combination) of overburdened buffering/clearance mechanisms, increased calcium flux via NMDA-type glutamate receptors or voltage-gated L-type and T-type Ca^{2+} channels, or exacerbated release from internal stores (Foster, 2007; Nunez-Santana et al., 2014; Oh et al., 2010, 2013; Thibault et al., 2007).

Accordingly, molecules that govern Ca^{2+} flux, buffering, and clearance are attractive targets for ameliorating cognitive aging and symptoms of AD. One repository of potential candidates is the smooth/agranular endoplasmic reticulum (ER), a voluminous organelle composed of fenestrated hypolemmal tubules (Cui-Wang et al., 2012; Peters, Palay, & Webster, 1991; Spacek and Harris, 1997; Terasaki, Slater, Fein, Schmidek, & Reese, 1994) involved in multiple cellular functions in neurons, including tight regulation of intracellular Ca^{2+} (Berridge, 2002). The ER maintains rigid control of internal Ca^{2+} levels by balancing

Ca^{2+} leak from internal and external sources with its transport to internal stores using pumps like the plasma membrane and sarco/endoplasmic reticulum Ca^{2+} -ATPases (PMCA and SERCA, respectively). These pumps ensure that free, cytosolic Ca^{2+} concentrations remain low (nM range), while simultaneously loading internal stores (mM range) for use in elementary or global Ca^{2+} signaling via ryanodine and inositol 1,4,5-triphosphate receptors (RyRs and IP₃Rs, respectively). Depletion of internal Ca^{2+} stores during such signaling triggers activation of capacitative calcium entry, an ER refilling mechanism moderated by interactions among ER-bound proteins (Berridge, 1995; Irvine, 1990; Putney, 1986).

The presenilin gene family (*PSEN1* and *PSEN2*) encodes one class of these ER-bound proteins, mutations in which are responsible for many forms of familial/heritable AD (Alzheimer's Disease Collaborative Group, 1995; Levey-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). Importantly, presenilins with AD-linked mutations produce perturbations in Ca^{2+} signaling and homeostasis (Chakroborty et al., 2012; Etcheberrigaray et al., 1998; Green et al., 2008; Guo et al., 1996; Nelson et al., 2007; Tu et al., 2006; Yoo et al., 2000; Zhang, Sun, Herreman, De Strooper, & Bezprozvanny, 2010). Moreover, there is compelling evidence that other AD-associated macromolecules (e.g., A β oligomers) are also sufficient to elevate cytosolic Ca^{2+} to levels that disrupt neuronal function (Busche et al., 2008, 2012; Demuro et al., 2005; Kuchibhotla et al., 2008; Stutzmann et al., 2006).

The underlying events that link aberrant ER signaling to AD pathogenesis, however, are not well understood. For example, blocking RyRs with dantrolene (Chakroborty et al., 2012; Oules et al., 2012; Peng et al., 2012; Zhang et al., 2010, 2015) or activating them with caffeine (Cao et al., 2009) both ameliorate AD pathogenesis and neuronal dysfunction. Similarly, blocking IP₃Rs with heparin normalizes Ca^{2+} signaling in transgenic mouse models of AD (Goussakov, Miller, & Stutzmann, 2010). Interestingly, each of these manipulations also produces store depletion *h* (*SDh*) plasticity in non-transgenic rodent hippocampal neurons (Ashhad, Johnston, & Narayanan, 2015; Clemens and Johnston, 2014; Narayanan, Dougherty, & Johnston, 2010). *SDh* plasticity is thought to be an adaptive response to ER stress, and involves an increase in the functional density of hyperpolarization-activated, cyclic nucleotide-gated cation-nonspecific (HCN) channels. The increased density of HCN channels reduces neuronal excitability via changes in both active and passive membrane properties, and is therefore thought to be a protective, metaplastic mechanism in response to abnormal ER signaling.

In this study, we describe age-related changes in supra- and sub-threshold voltage signaling in hippocampal CA1 pyramidal neurons from AD transgenic (ADTg) mouse lines that harbor mutations in the *APP* and *PSEN1* genes (5 $\times$ FAD and 3 $\times$ Tg lines; refs. (Oakley et al., 2006; Oddo et al., 2003) and another which bears mutations in only the *APP* gene (J20 line; ref. (Mucke et al., 2000)). We then identify the molecular substrate for the sub-threshold voltage changes in the ADTg mice as a perisomatic sequestration of a multimolecular complex comprised of HCN1 and its auxiliary subunit tetratricopeptide repeat-containing Rab8b interacting protein (Trip8b; refs. (Lewis et al., 2009; Piskorowski, Santoro, & Siegelbaum, 2011; Santoro et al., 2011; Santoro et al., 2009)). Finally, we identify ER signaling as the final common pathway in this AD-linked HCN channelopathy, and demonstrate that widely prescribed drugs targeted to the ER can restore HCN channel

function and its non-uniform, distally enriched distribution via pharmacological induction of *SDh* plasticity.

2. Materials and methods

2.1. Animals

A total of 225 mice were used. ADTg mouse lines used were 76 3×Tg mice (Oddo et al., 2003), 48 5×FAD mice (Oakley et al., 2006), 6 J20 mice (Mucke et al., 2000), and 2 ARTE10 mice (12-months of age and included in the array tomography experiments only (Rodriguez-Arellano, Parpura, Zorec, & Verkhratsky, 2016). We also used their age-matched, wildtype, littermate controls (n = 93). Group definitions are 1-month (age range 1–2 months old), 6-months (age range 5–7 months old), 12-months (age range 10–13 months old), and 24-months (age range 18–25 months old).

The 3×Tg transgenic line has three mutations related to autosomal dominant, familial forms of Alzheimer's disease: one mutation in the APP gene (KM670/671NL/Swedish), one in the tau gene linked to frontotemporal dementia (P301L), and one in the PSEN1 gene (M146V). The 5×FAD transgenic line has five Alzheimer's-linked mutations: three mutations in the APP gene (KM670/671NL/Swedish; 1716 V/Florida; and V7171/London) and two in the PSEN1 gene (M146L and L286V). The J20 transgenic line has two mutations in the APP gene (KM670/671NL/Swedish; V717F/Indiana). The ARTE10 transgenic line has one mutation in the APP gene (KM670/671NL/ Swedish) and one mutation in the PS1 gene (M146V). In all transgenic lines, whole-brain amyloid load is low and plaque deposits are absent in the first few months in the hippocampus. Consequently, we consider the 1–2 month old group as the baseline. By 4–8 months, however, all ADTg lines exhibit extracellular plaque deposits, reaching steadily increasing levels of A β _{1–42} by 10–12 months of age. Moreover, synaptic and cognitive impairments begin to emerge at this age range, indicating that neuronal integration is impaired. Thus, we examined 12-month old ADTg mice of all lines and their wild-type littermate controls. Very few homozygous 5×FAD mice live beyond ~14 months, so this was considered the oldest age for the 5×FAD line. Importantly, however, the 5×FAD, ARTE10, J20, and 3×Tg mice have very high amyloid plaque and A β _{1–42} loads at this age. 3×Tg mice exhibit peak amyloid levels around 15 months of age, but many live up to 25–26 months of age, so we used this transgenic line and their wild-type controls to assess the impact of chronological aging, and whether continually increasing amyloid and human tau deposition exacerbate neuronal function beyond the effects at 12-months of age. Importantly, increases in intraneuronal A β and amyloid load in hippocampal tissue of 3×Tg mice precede detectable levels of human tau and its phosphorylated isoforms. Thus, examination of 3×Tg mice at both the 12- and 24-month timepoints provides the opportunity to compare the impact of high amyloid load (at 12-months of age) to the dual impact of high amyloid load and high levels of human tau (at 24-months of age). Using this design, we desired to capture the impact of both aging and amyloidosis (and human tau deposition) on HCN channel function/expression and action potential output. Though we only examined two ARTE10 mice and their wildtype littermate controls in the array tomography experiments, we consider the validation of the AD-linked HCN1 channel mislocalization channelopathy an important observation.

All experiments were conducted and analyzed blind with respect to genotype and age, using protocols approved by the Rush University Institutional Animal Care and Use Committee.

2.2. Slice preparation and electrophysiology

Dorsal hippocampal slices were prepared as described previously (Dougherty et al., 2013; Neuman et al., 2015). Briefly, following deep anesthesia with isoflurane, mice were transcardially perfused with ice-cold, oxygenated, sucrose-based artificial cerebrospinal fluid (aCSF; in mM: sucrose 210, NaH_2PO_4 1.25, NaHCO_3 25, CaCl_2 0.5, MgCl_2 7, dextrose 7, ascorbic acid 1.3, sodium pyruvate 3) and decapitated. Brains were removed and hemisected along the longitudinal fissure, followed by an oblique cut near the occipital lobe, which produced the flat surface glued to the mounting block of the vibrating thermally controlled microtome (Microm 650 V; ThermoFisher Scientific). Slices of the dorsal hippocampus (315 μm) were obtained in ice-cold sucrose-based aCSF and then transferred to a holding chamber to recuperate for 25 min at 37 °C in oxygenated aCSF (in mM: NaCl 125, KCl 2.5, NaH_2PO_4 1.25, NaHCO_3 25, CaCl_2 2, MgCl_2 2, dextrose 10, ascorbic acid 1.3, sodium pyruvate 3), and at room temperature in the same solution thereafter. Slices were visualized using a fixed-stage, upright microscope (AxioExaminer; Carl Zeiss Microimaging, LLC) equipped with differential interference contrast videomicroscopy (NC-70; DAGE-WTI).

Whole-cell current-clamp recordings were obtained at least 5 min after break-in to whole-cell configuration using a Multiclamp 700B amplifier and pCLAMP data acquisition software (Molecular Devices). Slices in the recording chamber were continuously perfused with oxygenated aCSF and SR95531 (4 μM ; Tocris) and CGP55845 (1 μM ; Tocris) at 32–34 °C. Patch-pipettes were pulled from 1.5 mm outer diameter filamented, borosilicate pipettes to a resistance of 3–5 M Ω , and filled with internal solution containing (in mM): 115 K gluconate, 20 KCl, 10 Na_2PCR , 10 HEPES, 2 MgATP, 0.3 NaGTP, and 0.5% biocytin (pH = 7.3, ~285 mOsm). Series resistance and capacitance compensation were monitored continuously, with series resistances exceeding 30 M Ω leading to termination of the experiment. All recordings were taken at a common membrane potential, held at –67 mV.

Input resistance (R_N) was estimated as the slope of the current–voltage relationship using a family of 700 msec current injections in the subthreshold range (–50 pA to +50 pA in 10 pA steps). Rebound slope (RS) was determined using a family of hyperpolarizing 700–1000 msec current injections (–300 pA to –50 pA in 50 pA steps), as the slope of the relationship between steady state membrane potential and the amplitude of the posthyperpolarization rebound depolarization. Rheobase (i.e., the minimum current injection necessary to trigger 1 axonal action potential) was determined using a family of 1000 msec depolarizing currents in steps of 20 pA. After completion of each experiment, slices were immediately stored in 4% paraformaldehyde and processed for post-experiment visualization of dialyzed biocytin (using either 3,3'-diaminobenzidine or 1:300 streptavidin-conjugated AlexaFluor 488) within 3–5 days. Only CA1 pyramidal neurons that could be morphologically verified were included in the experiments.

2.3. Pharmacological manipulations

R_N, RMP, RS, and AP output were assessed using whole-cell patch-clamp physiology before and after bath-applied drugs, which involved perfusion through the recording chamber for 10 min followed by wash out for 15 min. These same parameters were examined using drugs dissolved in the internal pipette solution, at time = 0' and time = 25'. Bath applied drugs were the RyR blocker dantrolene (10 μ M; Tocris), the SERCA pump inhibitor cyclopiazonic acid (20 μ M; Tocris), the RyR agonist caffeine (10 mM; Sigma-Aldrich), the RyR blocker and beta and alpha adrenergic blocker carvedilol (10 μ M; M. Fill), and the partial RyR blocker ryanodol (10 μ M). Drugs applied intracellularly via the patch pipette were the IP₃R blocker heparin (1mg/ml) and the SERCA pump inhibitor thapsigargin (2 mM; Calbiochem).

2.4. Western blots

Mice were anesthetized with isoflurane and transcardially perfused with the sucrose-based aCSF used for patch-clamp experiments. The brain was immediately removed, hemisected, and cut to isolate the dorsal hippocampus. The hemispheres were then mounted on the cut surface and sliced at 300 μ m with a Microm 650 V vibratome (ThermoFisher Scientific, Waltham, MA) at 4 °C while immersed in oxygenated sucrose-based aCSF. Slices were immediately flash frozen in liquid nitrogen then placed over dry ice. The CA1 region was micro-dissected and placed in a cell lysis buffer (CLB) containing (in mM): 10 HEPES, 10 NaCl, 1 KH₂PO₄, 5 NaHCO₃, 5 EDTA, 1 CaCl₂, 0.5 MgCl₂, and HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA), and stored at -80 °C until use.

A subcellular fractionation protocol was used to obtain proteins found in the membrane as previously described (Guillemin, Becker, Ociepa, Friauf, & Nothwang, 2005; Simkin et al., 2015). Briefly, tissue was incubated over ice and homogenized with a Teflon-coated motorized homogenator. To restore isotonic conditions, 2.5 M sucrose was added at 0.1 vol. The sample was centrifuged at 8000 rpm (6000g) at 4 °C after which the supernatant containing the membrane, organelles, and cytosolic fractions was collected. The pelleted nuclear fraction was discarded. The supernatant was further centrifuged at 14,000 rpm (18,000g) 4 °C for 150 min. The supernatant containing the cytosolic fraction was collected while the pellet containing the membrane and organelle fraction was re-suspended in CLB containing protease and phosphatase inhibitors. Protein concentrations were determined using a Nanodrop Spectrophotometer (ThermoFisher Scientific).

Thirty micrograms of protein were reduced with BOLT LDS Sample Buffer and BOLT Sample Reducing Agent (NOVEX, Life Technologies), loaded into an 8% Bis-Tris gel (Invitrogen) and run in MOPS running buffer at 165 V for 45 min. The gel was then rinsed in water and transferred to a nitrocellulose membrane under wet transfer conditions using a Bolt Mini Blot Module wet transfer system (10 V for 60 min, NOVEX, Life Technologies). Following transfer, the membrane was briefly washed in PBS then incubated in acetic acid 7%/EtOH 10%. Total protein load was probed using a fifteen minute incubation in SYPRO Ruby (Life Technologies) followed by imaging on a BioRad QX4000 imaging system (Bio-Rad). The membrane was then rinsed in phosphate buffered saline (PBS) and blocked for 60

min with Odyssey Blocking Buffer (LI-COR Biosciences). The membrane was then incubated with anti-HCN1 antibody (Neuromab), and anti- β -actin antibody (Sigma-Aldrich) in Odyssey Blocking Buffer and 0.1% Tween20 on a rotator at 4 °C overnight.

Following three rinses in PBS with Tween-20 (PBST, 20 min each), the membrane was incubated with IR-conjugated secondary antibody (goat anti-rabbit, LI-COR Biosciences) in Odyssey Blocking buffer containing 0.1% Tween20 and 0.01% SDS for 40 min at room temperature. The membrane was then rinsed in PBST (2×10 min) and PBS (2×5 min) before being imaged on a LI-COR Odyssey Imager (LI-COR Biosciences). Multiple exposures levels were collected, and the most representative image was selected for analysis. Bands were visualized with NIH ImageJ software and normalized to total protein load and β -actin, then to WT littermate values.

2.5. Immunofluorescence array tomography

Dorsal hippocampal slices from the patch-clamp experiments were used to visualize HCN1 localization using immunofluorescence array tomography as described previously (Neuman et al., 2015). Briefly, slices were post-fixed in 4% paraformaldehyde immediately following patch-clamp experiments for 3–5 days, then bathed in 1:300 dilution of streptavidin-conjugated Alexa Fluor 488 (Life Technologies), microwave processed (Pelco BioWave Pro), and embedded in LR white (SPI Supplies). Slices were trimmed to a rectangle containing the recorded neuron and the surrounding CA1 region, and arrays of 70–150 serial ultrathin sections (67 nm) were mounted onto gelatin-subbed, high-precision coverslips (Aratome) using an ultramicrotome (Leica UC6) and a diamond knife (Diatome Histo Jumbo). Arrays were blocked, immunostained for HCN1 (Santa Cruz Biotechnology), rinsed, incubated in secondary Alexa Fluor 594 (Life Technologies), rinsed, and mounted with SlowFade Gold AntiFade with DAPI (Life Technologies). Images were acquired with a Zeiss AxioImager.M2 system equipped with an Axiocam MRm digital camera, AxioVision Software, and 63×/1.4NA Plan Apochromat oil-immersion objective lens. Mosaics were obtained with aid of custom-written plugins provided by Stephen Smith's laboratory. The middle 50 sections were imaged, aligned, cropped, deconvolved, thresholded, and analyzed using percent area fluorescence as the parameter of interest using Imaris software (Bit-plane).

2.6. Co-immunoprecipitation

Dorsal hippocampi were homogenized in ice-cold lysis buffer (N-PER™, ThermoFisher Scientific) with a protease inhibitor cocktail (ThermoFisher Scientific), using a glass Teflon homogenizer and incubated on ice for 10 min. Homogenates were centrifuged for 12 min at 12,000g (4 °C), after which supernatants were collected. The collected supernatants were divided into separate aliquots for each of the following conditions: input (total protein lysate, approximately 10% of total protein), anti-HCN1 immunoprecipitation, and non-specific goat IgG control immunoprecipitation. For immunoprecipitation, 435 μ g of protein was diluted in 400 μ L of lysis buffer and incubated overnight at 4 °C in a rotator with 2 μ g of polyclonal goat anti-HCN1 antibody (Santa Cruz Biotechnology) or 2 μ g of non-specific polyclonal goat IgG for control. 20 μ L of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were added to the mixtures and incubated for 2 hr at 4 °C on a rotator. Beads

were pelleted by centrifugation for 5 min at 1000g and washed with Cell Lysis Buffer (Cell Signaling). Beads were washed in this fashion a total of five times. Proteins were then eluted and boiled in Laemmli sample buffer and resolved by SDS-PAGE in a 3–8% Bis-Tris gel, and transferred to an iBlot® Transfer Stack, PVDF Membrane (ThermoFisher Scientific). Membranes were blocked for 30 min with PBS StartingBlock™ blocking buffer (ThermoFisher Scientific) and incubated overnight at 4 °C with polyclonal guinea pig anti-HCN1 (Shin and Chetkovich, 2007), monoclonal mouse anti-Trip8b (Antibodies Incorporated USA), and monoclonal mouse anti-β-Actin (Santa Cruz Biotechnology). Membranes were then washed four times for 5 min each in Tris-buffered saline/0.1% Tween (TTBS) and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature and washed four times for 5 min each in TTBS. The membranes were incubated for 3 min in SuperSignal™ West Dura (ThermoFisher Scientific), and visualized with the ChemiDoc™ Touch Imaging System (Bio-Rad). Blots were analyzed using ImageJ (National Institutes of Health) and normalized to loading control.

2.7. Immunogold electron microscopy

The distribution of HCN1 channels and Trip8b protein was examined using serial section, pre-embedding, silver-intensified, ultra-small immunogold electron microscopy as described previously (PESI-USIGEM; (Dougherty et al., 2013; Lörincz, Notomi, Tamas, Shigemoto, & Nusser, 2002; Notomi and Shigemoto, 2004). Briefly, mice were perfused with room temperature 0.9% saline, followed by acidic sodium acetate-buffered 2% paraformaldehyde/1% glutaraldehyde (pH = 6.0), then basic sodium borate-buffered 2% paraformaldehyde/ 1% glutaraldehyde (pH = 9.0). Slices (70 μm) were taken of the dorsal hippocampus, rinsed in Tris-buffered saline (TBS), exposed to 1% NaBH₄, rinsed in TBS, blocked, and then incubated in anti-HCN1 antibody (1:1000; (Shin, Brager, Jaramillo, Johnston, & Chetkovich, 2008). After rinsing in TBS, slices were blocked again and incubated in ultrasmall immunogold particles (Aurion, Electron Microscopy Sciences), followed by rinsing in TBS, fixation of immunogold particles in 2% glutaraldehyde in phosphate-buffered saline (PBS), silver enhancement using the R-Gent SE-EM Enhancement Kit (Aurion), then osmicated and cured in Araldite 502. Polymerized slivers of CA1 were then dissected from slices, re-embedded, and rotated orthogonal to the plane of sectioning. Arrays of serial ultrathin sections (64 nm, as estimated using Small's Method of Minimal Folds) were then collected using a Leica UC6 ultramicrotome and a diamond knife, followed by counterstaining in 5% aqueous uranyl acetate and Reynold's lead citrate. Images were obtained at 7500× or 10,000× magnification using a JEOL 1200EX transmission electron microscope or a Sigma HD VP scanning electron microscope in scanning-transmission (STEM) mode (Zeiss), respectively. The total number of dendrites analyzed in the PESIUSIGEM experiments was 360 and 500 dendrites with membrane-immunopositive signal for HCN1 in pSR and SLM, respectively. PESI-USIGEM experiments for Trip8b expression involved were based on 130 and 144 dendrites with membrane-immunopositive signal for Trip8b in pSR and SLM, respectively.

2.8. In vivo infusions of SDh plasticity inducers

Mice were anesthetized via inhalation using an isoflurane vaporizer (Vetamac) with O₂ pressure at ~700 mm and isoflurane flow set at ~2. Mice were then mounted onto a stereotaxic headholder (Stoelting), such that lambda and bregma were level with each other. Two small burr holes were then drilled above the CA1/subiculum border (coordinates: AP = 1.7, ML = 1.3, DV = 1.6), followed by counter-balanced infusion of 10 μ L of either aCSF vehicle or 20 μ M CPA (n = 1 12-month male 5 $\times$ FAD; 1 24-month old male 3 $\times$ Tg mouse; and 1 12-month old WT littermate control mouse) or 10 μ M carvedilol (n = 1 24-month old female 3 $\times$ Tg mouse) into CA1 using a Harvard Apparatus pump, a Hamilton microsyringe, and a 27 gauge stainless steel infusion cannula at a rate of 0.7 μ L/min. The infusion cannula was kept in place for 5 min after the infusion stopped. Mice were kept in the stereotaxic apparatus for a total of 25 min from the beginning of drug infusion (to approximate conditions used in patch-clamp experiments), and then immediately perfused and processed as described for immunogold electron microscopy. There was damage to tissue in the immediate vicinity of the infusion cannula (as determined by finding the cannula track in 70 μ m-thick sections). We took precautions such that we only re-embedded hippocampal slices with no visible cannula tracks, and with no damage at the ultrastructural level. The success of this precautionary approach can be seen in the electron micrographs from the infusion experiments, which exhibit healthy-looking dendrites with intact microtubules and normal diameters. The 27 gauge cannula size was chosen as it is small enough to limit damage, but large enough to avoid being clogged as it was being lowered into the hippocampus. The total number of immunopositive dendrites analyzed from these mice was 236 in pSR and 256 in SLM.

2.9. Statistical analyses

For all analyses, a *p* value < 0.05 was considered statistically significant. Asterisks in all figures indicate a significant difference at this *p* value, and all group means are represented as $\pm$ the standard error of the mean. For AP data, repeated measures analysis of variance (ANOVA) was used with time epoch as the repeated measure and AP number and normalized AP number as the dependent variable. For R_N, ISI frequency, RMP, RS, and sag data, one-way ANOVA was used. Correlations among sag, R_N, RS, and RMP were probed using Product-Moment correlations. Western blot, array tomography, immunogold electron microscopy, and immunoprecipitation data were compared using t-tests and ANOVA. The effect of pharmacological treatments were assessed using repeated measures ANOVA or t-tests. We found no differences among the different ADTg mouse lines during our analyses (i.e., all ADTg experiments yielded wildtype-like data at ages 1–2 months; and all ADTg mouse lines exhibited similar electro-physiological abnormalities and HCN1 mislocalization after about 10–12 months of age), so all ADTg lines were pooled for all statistical analyses, segregated only by age.

3. Results

3.1. Mouse models of Alzheimer's disease/amyloidosis

The four ADTg mouse lines we examined were chosen based on (i) their wide use among researchers; (ii) their increased amyloid loads at 12 months of age; and (iii) their different

life expectancies (Mucke et al., 2000; Oakley et al., 2006; Oddo et al., 2003; Rodriguez-Arellano et al., 2016). If our experimental observations were influenced by age only, then we would expect that the longest living of the mouse lines (the 3×Tg line) would show the most robust differences. If, however, chronological aging and amyloid interact, then a threshold amyloid load might be detected, such that long-term exposure (~10–12 months) to high amyloid loads might be sufficient to disrupt normal voltage signaling. Indeed, as described below, voltage signaling in the super- and sub-threshold range was disrupted as early as 6 months of age (in the 5×FAD line), and was uniformly disrupted in all transgenic lines by 12 months of age. Importantly, however, the channelopathy we describe could be rescued in all mouse lines, even among 24-month old 3×Tg mice, supporting the idea that pharmacological liberation of sequestered HCN1 channels is possible regardless of chronological age.

3.2. Exacerbated accommodation at perithreshold currents progressively worsens with age in CA1 pyramidal neurons of ADTg mice

A previous study found that the Ca^{2+} -sensitive AHP triggered by suprathreshold current injections was augmented as early as eight months of age in 5×FAD mice (Kaczorowski et al., 2011). One widely reported consequence of such an observation is exacerbated spike frequency adaptation or accommodation (Landfield and Pitler, 1984; Moyer, Thompson, Black, & Disterhoft, 1992, 2000). Using somatic patch-clamp recordings and comparatively smaller perithreshold current injections, we found that this was indeed the case in hippocampal CA1 pyramidal neurons from three different ADTg mouse lines (3×Tg, 5×FAD, and J20), but not their wildtype (WT) littermates at ages ranging from 1-month old to 24-months old (Fig. 1A–C). The exacerbated accommodation progressively worsened throughout chronological aging in the ADTg mice (Fig. 1A–C; one-way ANOVA on ADTg AP number in 2nd half of current injection, main effect of Age, $F_{(3,100)} = 2.79$, $p = 0.04$).

That this is detectable at current injections near the minimum necessary to trigger APs (and using smaller depolarizing current injections than those typically used in studies of post-burst AHP amplitudes) suggests that neurons in the ADTg mice may bear abnormalities other than increased AHPs and/or their underlying conductances. Indeed, despite WT-like *peri*-threshold AHP amplitudes (Fig. S1), AP amplitudes, AP thresholds, AP half-widths, rheobase currents (Table 1), and 1st-2nd AP interspike intervals (Fig. 1D), there were notable age-related changes in neurons from ADTg mice in two parameters that are heavily influenced by HCN channels (Biel, Wahl-Schott, Michalakis, & Zong, 2009; He, Chen, Li, & Hu, 2014; Pape, 1996; Robinson and Siegelbaum, 2003): input resistance (R_N) and resting membrane potential (RMP; Fig. 1D). As with increased accommodation, this pattern progressively worsened with age. The range of values in ADTg neurons was so large, in fact, that its collective impact produced population means near that for WT neurons, despite being derived from a much broader population of individual values (Fig. 1D). In other words, the broad range of values from ADTg neurons precluded a statistically significant difference, despite the presence of neurons with values far below or far above the WT range of values.

3.3. A Mixed HCN channelopathy emerges with age in ADTg mice

The consistent association between membrane properties that would be influenced by fluctuations in HCN channel function and progressive worsening of suprathreshold voltage signaling in the ADTg mice prompted us to probe subthreshold voltage signaling in the same neurons. HCN channels carry the I_h -current (I_h), which is a non-inactivating inward cation current. I_h counteracts hyperpolarization, leading to the characteristic “sag” in voltage sweeps during a hyperpolarizing current injection and a rebound depolarization when it ends (Chen, Aradi, Santhakumar, & Soltesz, 2002; Robinson and Siegelbaum, 2003; Shah, 2014). The amplitude of the rebound depolarization as a function of steady-state membrane potential during the hyperpolarizing current injection can be used to calculate rebound slope (RS; refs. Magee, 1998; Magee, 1999; Brager and Johnston, 2007; Lewis et al., 2011). Both of these subthreshold voltage signatures (sag and RS) are thus indices of HCN channel function and/or expression.

We estimated HCN channel function using a family of 700-msec hyperpolarizing current injections (–50 pA to –300 pA in 50 pA steps). Like AP output, values for both sag and RS were tightly distributed in WT mice at all ages (Fig. 2A–C). Sag and RS values for ADTg mice at 1-month of age were similar to WT mice, but by 6-months, CA1 pyramidal neurons in ADTg mice produced a wide range of values, some WT-like and others far outside the normal range (as defined by the neurons from WT mice; Fig. 2A–C). As with changes in accommodation, these changes worsened with age in ADTg mice. Sag and RS values were strongly correlated, supporting the notion that both estimate HCN channel function (Fig. 2D). Given the tight distribution of values for WT neurons and the broad distribution among ADTg neurons, we probed the relationships between RS and two neuronal parameters that are influenced by changes in HCN function/expression: R_N and RMP (Fig. 2E–G). In each case, we found links among the three values only in ADTg neurons. Indeed, the correlation between RMP and RS in ADTg mice was so strong that many neurons with gain-of-function channelopathies had resting membrane potentials near AP threshold (~–54 mV; Fig. 2G).

Interestingly, neurons near plaques or expressing intraneuronal 6E10 immunoreactivity had the same probability of being normal or channelopathic as neurons lacking plaques in their *ex vivo* slices and/or lacking 6E10 immunoreactivity (Fig. S2). Moreover, there were no notable morphological differences between WT and ADTg neurons at any age (Figs. S3–5). Thus, it is possible that circulating, non-plaque-associated amyloid-beta induces some molecular cascade that disrupts HCN channel regulation.

The co-emergence of this mixed HCN channelopathy with exacerbated accommodation suggests the possibility that both are precipitated by underlying cascades. The presence or absence of exacerbated accommodation, however, did not predict whether an individual neuron harbored an isoform of the mixed HCN channelopathy (data not shown). This indicates that the molecular mechanisms underlying the AD-linked changes in suprathreshold and subthreshold signaling are distinct. Given the principal role of HCN1 subunit of HCN channels in determining sag ratio and RS in CA1 pyramidal neurons (Brager and Johnston, 2007; Giocomo and Hasselmo, 2009; Kim, Chang, & Johnston, 2012; Lewis et al., 2011; Magee, 1998, 1999; Nolan et al., 2004), one possible explanation for the mixed HCN channelopathy is that some neurons in ADTg mice synthesize abnormally high

amounts of HCN1 (manifest as gain-of-function channelopathic neurons), some neurons synthesize a normal amount (normal), and others neurons synthesize an unusually low amount of HCN1 (manifest as loss-of-function channelopathic neurons). We used a combination of molecular biology and immunolocalization techniques to test this hypothesis.

In light of the observation that the HCN1 channelopathy and exacerbated accommodation emerges around 10–12 months of in all ADTg lines we examined (3×Tg, 5×FAD, and J20), we pooled the results in the subsequent (below) experiments across ADTg lines, such that we examined three age groups: (i) a 1-month old group, comprised of 3×Tg and 5×FAD mice and their wild-type littermate controls; (ii) a 12-month old group, comprised of 5×FAD, J20, and ARTE10 mice and their wild-type littermate controls; and (iii) a 24-month old group, comprised of 3×Tg mice and their wild-type littermate controls. As seen with the electrophysiological experiments, there was widespread uniformity in our observations, despite the genetic differences of the various ADTg lines.

3.4. Age-related perisomatic sequestration of HCN1 in aged ADTg mice

Studies in both human AD cases and their ADTg mouse models have found that numerous proteins involved in synaptic and dendritic function change relative to non-cognitively impaired cases/WT mice at both the single cell and population levels (Cochran, Hall, & Roberson, 2014; Gaiteri, Mostafavi, Honey, De Jager, & Bennett, 2016; Henstridge, Pickett, & Spires-Jones, 2016; Mufson et al., 2016). We first probed for HCN1 by western blot in enriched CA1 membrane fractions to estimate expression levels in 1–24-month old WT and ADTg mice (Fig. 3A). Whether expression levels were normalized to β -actin or total protein (using SYPRO Ruby), similar levels of HCN1 protein were found in CA1 homogenates from WT and ADTg mice at all ages (Fig. 3B).

It is possible that gain-of-function channelopathic neurons over-produce HCN1, which offsets the dearth of HCN1 synthesized by loss-of-function neurons, leading to no net change in total HCN1 protein load. To evaluate this possibility, we used the super-resolution light microscopy technique immunofluorescence array tomography (iAT) to immunolocalize HCN1 in hippocampal region CA1, obtained from excisions of slices used in the patch-clamp experiments. HCN1 has a nonuniform distribution in hippocampal region CA1, with high intensities in the pyramidal cell layer that fade until an abrupt increase in signal in the distal dendrites of stratum lacunosum-moleculare (SLM; (Dougherty et al., 2013; Lörincz et al., 2002; Notomi and Shigemoto, 2004). This so-called distal enrichment of HCN1 was detectable using iAT in CA1 of WT mice at 1-, 12-, and 24-months of age (Fig. 3C,D). HCN1 also exhibited distal enrichment in 1-month old ADTg mice (Fig. 3C,D). By 12 months of age, however, the distal enrichment in ADTg mice disappeared, such that there was little difference in immunofluorescence between the dendrites of SLM and the more proximal dendrites in stratum radiatum (SR; Fig. 3C,D). These data are inconsistent with the notion that the Western blot experiments can be explained by offsetting HCN1 synthesis levels in CA1 pyramidal neurons of ADTg mice because the iAT experiments show evidence for a near-global loss of HCN1 distal enrichment in the different ADTg mouse lines (3×Tg, 5×FAD, J20, as well as ARTE ADTg mouse lines).

The possibility remained, however, that the gain-of-function neurons, despite the loss of HCN1 distal enrichment had higher RS values because the proximal dendrites showed abnormally high expression levels for HCN1. The iAT experiments did not support this explanation, but the gold standard for determining compartment-specific channel expression is immunogold electron microscopy. To test this hypothesis, we used serial section pre-embedding, silver-intensified, ultrasmall, immunogold electron microscopy to estimate membrane expression of HCN1 channels in WT and ADTg mice at 1-, 12-, and 24-months of age (Fig. 3F). As with the iAT experiments, distal enrichment of membrane HCN1 expression was readily detectable in WT mice of all ages (Fig. 3F–H). Also consistent with the iAT experiments, distal enrichment of HCN1 was present at 1-month of age in the ADTg mice, but not at 12- or 24-months of age (Fig. 3F–H). Importantly, the densities of cytoplasmic immunogold particles were similar in the dendrites of SR and SLM in WT and ADTg mice (Fig. S6), indicative of a specific loss of HCN1 channels from the membrane of the distal dendrites in SLM. Taken together, these data are consistent with the idea that HCN1 channels are appropriately trafficked to the distal dendrites early in the lives of ADTg mice, but that by 10–12 months of age, an AD-linked pathogenic interaction (or lack of interaction) causes many of them to be sequestered perisomatically in the cytoplasm.

3.5. HCN1 is sequestered as a multimolecular complex with Trip8b in aged ADTg mice

Proper trafficking and gating of HCN1 channels in CA1 pyramidal neurons rely heavily on protein–protein interactions with the auxiliary subunit Trip8b (Han et al., 2016; Hu et al., 2013; Lewis et al., 2009; Piskorowski et al., 2011; Santoro, Wainger, & Siegelbaum, 2004, 2009, 2011). One possible explanation for the loss of distal enrichment of HCN1 channels in ADTg mice is that their interaction with Trip8b is disrupted, thereby obstructing the ability to deliver HCN1 to the distal dendrites. We used co-immunoprecipitation experiments to test this hypothesis by pulling down HCN1 and probing for Trip8b in dorsal hippocampal homogenates from 12- and 24-month old WT and ADTg mice (Fig. 4A). Neither HCN1 protein load, Trip8b protein load, nor the amount of Trip8b that co-immunoprecipitated with HCN1 was different between WT and ADTg mice (Fig. 4B), indicating that the protein–protein interactions critical for distal enrichment are intact despite its absence in ADTg neurons.

Immunolocalization studies have shown that the distribution of Trip8b, like HCN1, exhibits distal enrichment (Piskorowski et al., 2011; Santoro et al., 2004; Shin et al., 2008). We used serial section immunogold electron microscopy to determine whether distal enrichment of Trip8b, like that of HCN1, was present in WT mice, but absent in ADTg mice (Fig. 4C). Hippocampal region CA1 showed robust distal enrichment of membrane-bound immunogold particles for Trip8b at both 12- and 24-months of age in WT mice, but no such enrichment in ADTg mice of the same ages (12-months: J20 and 5×FAD lines; 24months: 3×Tg line). These data together indicate that many HCN1 channels in aged ADTg mice are sequestered perisomatically (or at least not in the distal dendrites) in the cytoplasm of CA1 pyramidal neurons as a multimolecular complex with Trip8b.

3.6. Inducing SDh plasticity rescues HCN channel function in ADTg neurons

That HCN1 and Trip8b protein loads and their interactions were intact in aged ADTg mice, despite the absence of their distal enrichment, is consistent with the idea that channelopathic and normal CA1 pyramidal neurons differed not in protein load, but in membrane-bound channel expression. We tested this idea using patch-clamp recordings and drugs that target the ER, which should trigger an ER stress response like SDh plasticity (Fig. 5A–C). Consistent with the idea that HCN1 channels are available but sequestered in pyramidal neurons of ADTg mice, SDh plasticity (using application of 20 μ M of the SERCA pump inhibitor cyclopiazonic acid; CPA) increased RS, rescuing even loss-of-function channelopathic neurons (Fig. 5B,C). Importantly, SDh plasticity could be induced using a variety of approaches to disrupt ER signaling including blocking ryanodine receptors with 10 μ M dantrolene, 10 μ M carvedilol, or 10 μ M ryanodol; agonizing them with 10 mM caffeine; blocking IP₃Rs with 1 mg/ml heparin in the patch-pipette; or depleting internal Ca²⁺ stores with 2 μ M of the non-competitive SERCA pump inhibitor thapsigargin in the patch-pipette (Figs. 5–7; Fig. S7). These observations support the idea that SDh plasticity and the accompanying exacerbation of accommodation are consistent responses to ER stress (see also Fig. 7). Importantly, even though ER manipulations increased HCN channel function in ADTg mice, such manipulations would be expected to reduce neuronal function in WT mice because of the consequent reduction in AP output.

3.7. In vivo infusions of SDh plasticity inducers restores HCN1 distal enrichment in aged ADTg mice

The mechanisms triggered by ER manipulations that augment RS are not well understood, but SDh plasticity-induction (using CPA) in dorsal CA1 pyramidal neurons increases dendritic HCN channel function in adult rats (Clemens and Johnston, 2014). It is conceivable that SDh plasticity induced by our ER manipulations rescued HCN channel function by restoring distal enrichment of HCN1 channels. To test this hypothesis, we infused either 20 μ M CPA or 10 μ M carvedilol (or vehicle) in vivo into the dorsal hippocampal CA1 region of anesthetized 12–24-month old ADTg mice, and then estimated dendritic HCN1 expression using serial section immunogold electron microscopy in the hemisphere receiving drug infusions and the hemisphere that received only vehicle infusions (Fig. 8A–D). As expected, distal enrichment in these aged ADTg mice was absent in the hemisphere receiving vehicle infusions (Fig. 8B,D). Remarkably, distal enrichment of membrane-bound HCN1 was restored to WT-like levels in CA1 of the hemisphere that received either CPA or carvedilol infusions (Fig. 8B,D). Consistent with the notion that perisomatically sequestered HCN1 channels are rapidly trafficked to the proximal and distal dendrites by manipulating ER signaling, CPA/carvedilol treatment on average increased both membrane and cytoplasmic particle numbers (per dendrite) by ~150% in the proximal dendrites of stratum radiatum (pSR) and by ~300% in the distal dendrites of SLM (Fig. 8B–D). These same observations were made in WT hippocampus after infusion of CPA, followed by immunogold electron microscopy for HCN1 (Fig. S8).

4. Discussion

Using functional, ultrastructural, immunocytochemical, molecular, and pharmacological approaches, we show that CA1 pyramidal neurons from numerous ADTg mouse lines harbor a progressive set of voltage signaling pathologies that emerges between 4 and 12 months of age: exacerbated accommodation and a reversible HCN mislocalization channelopathy. We further show that in older ADTg mice, HCN1 channels are sequestered in complex with the HCN1 auxiliary subunit Trip8b, and that ER signaling is the final common pathway for the aberrant supra- and sub-threshold voltage signaling. Finally, we used *in vivo* SDh plasticity induction in aged ADTg mice to show that ER manipulations rapidly increase HCN1 expression levels in the membrane and cytoplasm of proximal (~150% increase) and distal (~300% increase) dendrites, which consequently rescues the mislocalization channelopathy. Together then, our experiments show that AD-linked genetic mutations, including those limited to the APP gene (i.e., the J20 ADTg line), are sufficient to generate neuronal signaling abnormalities over chronological aging, at least one of which can be rapidly rescued by liberation and delivery of HCN1 channels to their proper locations in the distal dendrites.

Synapses have long been a target of inquiry with regard to the cognitive deterioration that helps define AD (e.g., refs. Scheibel, Lindsay, Tomiyasu, & Scheibel, 1975; DeKosky and Scheff, 1990; Terry et al., 1991). Our experiments support an expansion of the amyloid cascade hypothesis of AD (Allsop et al., 1988; Hardy and Higgins, 1992; Selkoe, 1991), wherein synapses and the dendritic ion channels with which they interact are targets of overlapping molecular pathogens (Cochran et al., 2014; Hall et al., 2015; Mucke and Selkoe, 2012; Palop et al., 2007; Siskova et al., 2014). For example, we previously showed that the 5xFAD mouse line experiences massive synapse loss in SLM around 12-months of age, which appears to be partially offset by an increase in the expression of AMPA-type receptors among the remaining synapses (Neuman et al., 2015). We now show that 5xFAD (and J20 and ARTE10) mice at this same age also show a global loss of HCN1 enrichment in this same region, establishing that synapto-pathogenesis is co-morbid with HCN1 mislocalization. Interestingly, CA1 pyramidal neurons from J20 mice as young as 3 months of age show dendritic signaling abnormalities linked to reduced expression of Kv4.2 channels (Hall et al., 2015), a voltage-gated ion channel also enriched in the distal dendrites (Hoffman, Magee, Colbert, & Johnston, 1997; Kerti, Lorincz, & Nusser, 2012). Thus, accumulating evidence suggests that the menagerie of ion channels that confers to cortical pyramidal neurons their elegant integrative abilities is dismantled in an age-related, progressive manner in ADTg mice. Importantly, however, our *in vivo* infusions of SDh plasticity inducers show that HCN1 channels can be rapidly trafficked to the distal dendrites to help restore the disrupted assembly of important voltage-signaling molecules. This may signal that other molecules and ion channels are also mislocalized perisomatically in ADTg mice (e.g., Kv4.2 channels), yet available for pharmacological liberation. Importantly, we observed HCN channel plasticity using a variety of pharmacological manipulations that would induce ER stress, including ryanodine receptor antagonists (dantrolene, carvedilol, and ryanodol), store depleting agents (CPA and thapsigargin), and blockade of IP₃Rs via heparin. We refer to the HCN channel plasticity as SDh plasticity, but perhaps ER stress-

induced HCN plasticity is a more accurate term. More work is needed to determine whether there is a distinction in the plasticity induced by the different ER manipulations.

Of importance, the mislocalization and dysfunction of HCN1 in CA1 pyramidal neurons from ADTg mice we chronicle here is not without precedent. Rather, numerous central nervous system disorders are associated with HCN1 abnormalities in humans or their animal models including Fragile X Syndrome (Brager, Akhavan, & Johnston, 2012; Kalmbach, Johnston, & Brager, 2015), autism spectrum disorders (Yi et al., 2016), schizophrenia (Lencz and Malhotra, 2015), depression (Kim et al., 2012; Lewis et al., 2011), Parkinson's disease (Chan et al., 2011), cognitive aging (Arnsten and Jin, 2014; Wang et al., 2011), and neuropathic pain (Santello and Nevian, 2015). One of the most studied neurological disorders associated with ion channelopathies is epilepsy (Dubé, Brewster, Richichi, Zha, & Baram, 2007; Lerche et al., 2013; Poolos and Johnston, 2012). Experimental induction of status epilepticus using kainic acid or pilocarpine injections initially upregulates perisomatic HCN1 expression and function, but is quickly followed by massive diminution of dendritic h-channel function and redistribution of HCN1 to the perisomatic cytosol (Jung et al., 2007, 2011; Shah, Anderson, Leung, Lin, & Johnston, 2004; Shin and Chetkovich, 2007; Shin et al., 2008). Similar to the results reported here (Figs. 3 and 4), the sequestered HCN1 is in a multimolecular complex with Trip8b (Shin and Chetkovich, 2007). Interestingly, as in ADTg mice (Hall et al., 2015), seizure induction also generates a channelopathy linked to decreased availability of Kv4.2 channels (Bernard et al., 2004). So, these neurological disorders, as modeled in animals, are linked by an inability to maintain the normal localization of voltage-gated ion channels that heavily influence synaptic integration. Future work is needed to determine if brain-bank, biopsy, or resection tissue from human cases show immunocytochemical evidence consistent with the animal work.

Some important questions remain unresolved by the current study. First and foremost is the question of whether the present findings have relevance to AD, and if so if its relevance is limited to the familial forms of the disease modeled genetically in the ADTg mice. Given that the mislocalization channelopathy was observed in the 3×Tg, 5×FAD, and J20 ADTg lines (and then validated in two ARTE10 ADTg mice), it is likely that there is at least some relevance. With regard to idiopathic versus familial forms of AD, the questions remain unanswered. Future work on brain bank tissue will provide the most insight into this question.

Second, where are the HCN1 channels that we are referring to as “sequestered,” which are then pharmacologically liberated by ER manipulations? There was no apparent deposition or clustering of immunogold particles in the perisomatic regions, nor was it evident in the array tomography experiments. We also quantified expression in the basal dendrites using immunofluorescence array tomography, but again failed to find an aberrant increase in expression of HCN1 in the ADTg mice (data not shown), which argues against HCN1 channels being inappropriately trafficked to other dendritic compartments. The most parsimonious explanation for our findings, collectively, is that HCN1 channels are mislocalized and sequestered in the cytoplasm with Trip8b. This protein–protein linkage then enables this multimolecular complex to be rapidly trafficked to the membrane of both distal and proximal dendrites, during both bath application and intrahippocampal infusions

of the drugs. There is the possibility, however, that CA1 pyramidal neurons show a cell-type specific downregulation of HCN1 synthesis, which is offset by an equally cell-type specific upregulation of HCN1. Both interneurons (Lupica, Bell, Hoffman, & Watson, 2001; Maccaferri and McBain, 1996) and non-neuronal cells like glial cells (Honsa et al., 2014) express HCN1. We did not observe any aberrantly high expression of either HCN1 or Trip8b on non-spiny dendrites, which argues against the possibility of the former upregulating HCN1 expression. The possibility remains that astrocytes, particularly reactive astrocytes that form an annulus around amyloid deposits and dystrophic neurites (Rodriguez-Arellano et al., 2016), upregulate their expression of HCN1 in a manner that offsets the CA1 pyramidal neuron cell-type specific downregulation. We probed the immunogold HCN1 tissue from both WT and the ADTg strains for reactive astrocytes, which are notable by their prominent neurofilaments in the cytoplasm of their astrocytic processes. They were exceedingly rare in the WT mice, but could be found, and were indeed immunopositive for HCN1. Conversely, reactive astrocytes in the ADTg tissue were found in the vicinity of dystrophic neurites throughout CA1, as well as in the alveus and basal and apical dendritic regions of CA1 in the ADTg mice (Fig. S9). Moreover, their expression levels of HCN1 at the immunogold level were extremely high (Fig. S9), perhaps supporting the notion that the increased presence of reactive astrocytes due to AD pathology in the ADTg was offsetting a cell-type specific reduction in HCN1 expression in CA1 pyramidal neurons. This notion, however, would not explain why HCN1 function and expression in CA1 pyramidal neurons in the ADTg mice can be returned to normal levels after ER manipulations on the time-scale of ~25 min. Clearly, future work with regard to interactions between neuronal and non-neuronal cells in ADTg mice and Alzheimer's disease brain tissue is needed.

Third, if persistent ER stress is present in the ADTg mice, why is *SDh* plasticity not engaged persistently at baseline in their neurons? There are at least two answers. First, the molecular cascades that normally link *SDh* plasticity to ER stress might be disconnected or weakened. Only by experimentally inducing *SDh* plasticity is the new, heightened threshold for plasticity triggered. Secondly, perhaps there is constitutive cycling of *SDh* plasticity from an on-phase to an off-phase. At some point in time, according to this possibility, every CA1 pyramidal neuron might have a gain-of-function perisomatic HCN channelopathy that dissipates with time, returning HCN function back to within WT values, which is then subsequently run-down toward a loss-of-function HCN channelopathy. Perhaps this cycle from gain-of-function to normal to loss-of-function constitutively oscillates and the neurons recorded in the present study were simply electrophysiological snapshots of neurons at different points of this cycling. Clearly, future work is needed to clarify these notions and distinguish between them.

And, fourth, is ER stress, calcium dysregulation, or unregulated *SDh* plasticity responsible for the exacerbated accommodation in the older ADTg mice? The pharmacological experiments (Figs. 5–8) suggest that ER stress and/or the ensuing calcium dysregulation is sufficient to increase both accommodation and HCN function. The current conducted by HCN channels, I_h , regulates accommodation in several cell types, however (Aponte, Lien, Reisinger, & Jonas, 2006; Neuhoff, Neu, Liss, & Roeper, 2002). So, it remains a possibility that the HCN channelopathy alone could drive changes in accommodation. Additionally, it is possible that HCN channels alone are not responsible for the observed range of values for

resting membrane potential and input resistance. Indeed, these parameters are governed by interaction between HCN channels and other K⁺-selective channels (e.g., Day et al., 2005). Moreover, the source of the calcium that is being dysregulated is not known. We presume that soluble amyloid-beta oligomers are involved, which can recruit NMDA receptor signaling that causes oxidative stress in neurons (De Felice et al., 2007) and also activate mGluR signaling pathways, including those involved in Ca²⁺ release (Renner et al., 2010; Um et al., 2013). Given that both NMDA receptor signaling (Campanac, Daoudal, Ankri, & Debanne, 2008) and mGluR-dependent Ca²⁺ release (Brager and Johnston, 2007) can drive HCN channel plasticity, it is possible that signaling via both receptor pathways may contribute to the HCN channelopathy observed here (and potentially its pharmacological rescue). So, there are likely maladaptive interactions or weaker interactions among the constellation of voltage-gated channels on the somatic and dendritic membrane of ADTg mice, particularly as they age. Again, more work is needed to address these important notions.

More information is also needed to understand the role of PS1, APP, and APP cleavage products (e.g., A β monomers and oligomers) in the supra- and sub-threshold voltage signaling abnormalities because our experiments on J20 mice show that APP mutations are sufficient to generate both (Figs. 1–4). Our experiments suggest, however, that these two are separable because most neurons in aged ADTg mice exhibited exacerbated accommodation, yet produced accompanying RS values that ranged from loss-of-function channelopathic to normal to gain-of-function channelopathic (Figs. 2, 5–7). The former observation replicates earlier observations in the Tg2756 and 5 $\times$ FAD ADTg mouse lines (Kaczorowski et al., 2011; Ohno et al., 2004), where the age-related emergence of reduced neuronal excitability was associated with changes in the calcium-activated potassium current underlying the slow post-burst. Importantly, *SDh* inducers increased HCN channel function even in such neurons with preexisting hypoexcitability (e.g., Fig. 6), indicating that HCN channel-associated cellular responses to ER manipulations are intact in neurons harboring alterations in currents other than those carried by HCN channels.

Of note, the drug treatments implemented in our experiments have the dual benefit of restoring neuronal integrative potential via rapid expression of HCN1 channels in the distal dendrites while also allowing (hypoexcitable) neurons with exacerbated accommodation to rest at voltages close to axonal action potential threshold (Fig. 2D). Though speculative, this two-hit impact could be considered an adaptive form of metaplasticity and may help explain the improvements of AD pathogenesis and cognitive impairments observed after feeding ADTg mice dantrolene (Chakroborty et al., 2012; Goussakov et al., 2010; Oules et al., 2012; Peng et al., 2012; Zhang et al., 2015), caffeine (Cao et al., 2009), or carvedilol (Arrieta-Cruz, Wang, Pavlides, & Pasinetti, 2010; Wang et al., 2011; see also ref. Howlett, George, Owen, Ward, & Markwell, 1999). Whether the drug treatments used in the present experiments shift the threshold for inducing either intrinsic or synaptic plasticity is unknown, so future experiments are needed to determine if the two-hit impact of the pharmacological manipulations is indeed conferring to neurons metaplasticity that positively impacts plasticity thresholds and/or learning and memory. Importantly, carvedilol is a potent RyR blocker (Zhou et al., 2011) that rescues HCN channel function and is a widely

prescribed beta-blocker for patients at risk for congestive heart failure (DiNicolantonio, Lavie, Fares, Menezes, & O’Keefe, 2013; Packer et al., 1996).

Taken together, our results indicate that co-opting what appears to be an ER stress response restores HCN signaling and localization in hippocampal neurons in ADTg mice. Moreover, our work –and that of others– implies that AD patients and experimental animal models of AD pathogenesis (and perhaps other neurological disorders) may benefit from pharmacological interventions similar to those described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Progressive variability in intrinsic membrane parameters accompanies age-related exacerbation of accommodation in ADTg neurons.

(A) A family of depolarizing current injections was used to trigger action potentials (APs) in CA1 pyramidal neurons from WT (black voltage traces) and ADTg (3xTg = purple voltage traces; 5xFAD = red voltage traces; J20 = green voltage traces) mice at 1-, 6-, 12-, and 24-months of age, ranging from the minimum necessary to trigger a single AP (rheo) to rheo + 100 pA. Scale bar = 40 mV/1 sec. (B) Heat plots of the firing rate during rheo + 100 pA sweeps in 100 msec bins, normalized to peak firing rate. Each row displays data from a single neuron; mouse line for neurons is indicated along the left vertical edge of each heat

plot. (C) Raw and normalized AP number as a function of time as 1st half/2nd half of the current injection (left) or throughout the duration of the injection (right) at the various ages studied. (D) Input resistance (RN), resting membrane potential (RMP), and interspike interval (in Hz) between the 1st and 2nd APs at rheo + 100 pA. In all panels, asterisks indicate significant differences at $p < 0.05$. White circles = data from WT neurons; purple circles = 3×Tg neurons; red triangles = 5×FAD neurons; green squares = J20 neurons. Similarly colored bars represent the group means. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. A mixed HCN channelopathy emerges and worsens with age in hippocampal CA1 pyramidal neurons from ADTg mice.

(A) Voltage sweeps in response to a family of hyperpolarizing currents that was injected into CA1 pyramidal neurons from mice at the ages listed. Scale bar = 10 mV/500 msec. (B) Rebound slope (RS) in response to the hyperpolarizing current injections. (C) Membrane sag ratio (Sag) in response to the hyperpolarizing current injections. (D) Scatterplot showing the strong correlation between Sag and RS. (E) Scatterplot showing the relationship between input resistance (R_N) and RS. (F) Scatterplot of R_N versus resting membrane potential (RMP). (G) Scatterplot showing the strong relationship between RMP and RS specific to neurons from ADTg mice. In all panels, asterisks indicate significant differences at $p < 0.05$. Color-coded voltage traces are as in Fig. 1. White circles = data from WT neurons; purple circles = 3xTg neurons; red triangles = 5xFAD neurons; green squares = J20 neurons. Similarly colored bars represent the group means. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. ADTg mice harbor an age-related HCN1 mislocalization channelopathy.

(A) Western blots of HCN1 protein, β actin, and Sypro from WT and the ADTg lines at the ages listed. (B) Group means for HCN1 protein normalized to either β actin or Sypro at the ages shown. Black bars = WT data; red bars = ADTg data. (C) Summed immunofluorescence array tomographic projections for HCN1 from sections of hippocampal CA1 for the ages and mouse lines listed. pcl = pyramidal cell layer; sr = stratum radiatum; slm = stratum lacunosum-moleculare; white/red = HCN1 puncta; blue = DAPI puncta. Scale bar = 75 μ m for monochrome images; 25 μ m for RGB images; pSR (bottom row) and SLM

(top row). (D) Percent area fluorescence for HCN1 immunosignal in CA1 stratum radiatum (sr) or stratum lacunosum-moleculare (slm). Black = WT mouse data; red = ADTg mouse data. (E) The ratio of immunosignal in SLM to that in SR. Colors are the same as in (D). (F) Immunogold electron micrographs in proximal stratum radiatum (pSR; top row) and SLM (bottom row). Individual electron micrographs are labeled with the age-group and mouse line (WT = wildtype; 3× = 3×Tg; 5× = 5×FAD; J20 = J20). Scale bar = 0.5 μm in taller panels and 0.25 μm in shorter ones. (G) Membrane-bound immunogold particle density per dendrite, expressed relative to μm^2 of dendritic surface area for the age-groups and mouse lines. (H) Ratio of membrane-bound immunogold particle density per dendrite in SLM relative to that in SR. Colors in (G,H) correspond to groups lines as in (D,E). Group data are mean $\pm$ S.E.M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. HCN1 and Trip8b are sequestered as a multimolecular complex in ADTg neurons.

(A) Western blots of input, HCN1- and mock-IP (IgG) material from 12-month old wild-type (WT) and littermate 5xFAD mice (left) and 24-month old WT and littermate 3xTg mice (right). (B) Pixel density of the immunoblots normalized to WT values for HCN1, Trip8b, and Trip8b co-immunoprecipitated with HCN1. (C) Immunogold electron micrographs of Trip8b immunoreactivity in proximal stratum radiatum (pSR) and stratum lacunosum-moleculare (SLM) for the age groups and mouse lines indicated. Scale bar = 0.5 μ m in taller panels and 0.25 μ m in shorter ones. (D) Membrane-bound immunogold particle density per dendrite, expressed relative to μ m² of dendritic surface area for WT (black) and ADTg (red) mice. (E) Ratio of membrane-bound immunogold particle density per dendrite in SLM relative to that in SR. Colors in (B) correspond to groups as in (D,E). Group data are mean $\pm$ S.E.M. White circles = data from WT neurons; purple circles = 3xTg neurons; red triangles = 5xFAD neurons; green squares = J20 neurons. Similarly colored bars represent the group means. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Pharmacological manipulation of ER signaling in ADTg neurons enhances h-channel function, even in loss-of-function channelopathic neurons.

(A) Experimental timeline used in the experiments shown in Figs. 5–7. Break-in to whole-cell configuration at timepoint a (0'); measure intrinsic parameters and rebound slope at timepoint b (5' after whole cell configuration); begin bath application of drug at timepoint c (for 10'); stop bath application of drug at timepoint d (10' after timepoint c); measure intrinsic parameters and rebound slopes in response to drug application (25' after the beginning of bath application of drug, 15' minutes after bath application ends; end recording at timepoint f. (B) Voltage sweeps in response to hyperpolarizing current

injections before (red) and after (blue) bathing the slices in drugs that manipulate ER signaling. Numbers correspond to the neuron's rebound slope (RS). Sweeps (from top to bottom) derive from a 12-month old 5×FAD mouse neuron treated with cyclopiazonic acid (CPA), a 12-month old 5×FAD mouse neuron treated with CPA, a 12-month old 5×FAD mouse neuron treated with CPA, a 24-month old 3×Tg mouse neuron treated with caffeine, and a 12-month old 5×FAD mouse neuron treated with dantrolene as detailed in Experimental Procedures. Scale bar = 10 mV/500 msec. (C) Summary data from all ADTg mouse experiments showing RS values before and after (blue) manipulating ER signaling. Green shading represents the range of RS values recorded from wild-type neurons Fig. 2. Color-coded voltage traces are as in Fig. 1. Purple circles = data from 3×Tg neurons; red triangles = data from 5×FAD neurons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Numerous ER manipulations induce SDh plasticity in ADTg neurons.

(A) Voltage sweeps before (black for wild-type neurons; red for ADTg neurons) and after (blue) drug treatment during depolarizing currents sufficient to trigger at least one axonal action potential (rheo) and rheo + 100 pA (+100) for the age groups and mouse lines indicated. Scale bar = 50 mV/250 msec. (B) Voltage sweeps before (black for wild-type neurons) and after (blue) drug treatment during a family of hyperpolarizing currents for the age groups and mouse lines indicated. Scale bar = 10 mV/500 msec. Heparin was added to the internal pipette solution, so only post-treatment data were obtained. Color-coded voltage

traces are as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. ER manipulations that induce SDh plasticity partially abrogate differences between WT and ADTg neurons.

(C) Group data from the WT (black) and ADTg (red) treated neurons showing that many group differences in the parameters listed prior to drug treatments (pre) disappear subsequent to them (post). Asterisks denote repeated measures effects of drug exposure; double-asterisks denote repeated measures X genotype interaction. All significant differences are at $p < 0.05$; group data presented as average $\pm$ SEM. CPA data derive from 5 wt, 4 3×Tg, and 5 5×FAD neurons; RyR block data derive from 3 wt and 4 5×FAD neurons; caffeine data derive from 3 wt, 3 3×Tg, and 2 5×FAD neurons; heparin data derive from 5 wt and 4 3×Tg neurons. Experimental design is as depicted in Fig. 5A. Heparin was included in the internal solution of the patch pipette, and data was collected 30 min after break-in to whole-cell patch-clamp configuration. Single asterisks denote significant effect of repeated measure (before vs. after drug application); double asterisks denote a repeated measure X genotype interaction, meaning group differences before were no longer present after drug application. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Intrahippocampal infusions of SDh plasticity inducers rescue the HCN1 mislocalization channelopathy in ADTg mice.

(A) Experimental design matched the timeline of patch-clamp experiments, involving 10-minute infusion times, followed by 15 min of waiting time, and then perfusion for immunogold electron microscopy. (B) Membrane-bound immunogold particle number (per dendrite; each symbol represents a single dendrite) in the proximal stratum radiatum (psr) or stratum lacunosum-moleculare (slm) of CA1 in the hemisphere receiving infusions of vehicle (veh; red or purple) or the hemisphere receiving infusions of cyclopiazonic acid (CPA; blue) or carvedilol (carv; blue). Asterisks denote significant differences at $p < 0.05$.

Red bars denote means for 5×FAD tissue; purple bars denote means for 3×Tg tissue. (C) Immunogold electron micrographs in proximal stratum radiatum (pSR; top two rows) and SLM (bottom two rows) from CA1 infused with vehicle (veh) or *SDh* plasticity inducers (CPA and carv). The micrographs in the left column were taken from a 12-month old 5×FAD mouse; the micrographs in the middle column were taken from a 24-month old 3×Tg mouse; the micrographs in the right column were taken from another 24-month old 3×Tg mouse. Scale bar = 0.5 μ m in taller panels and 0.25 μ m in shorter ones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Action potential parameters in WT and ADTg mice.

Parameter	1-month WT	1-month ADTg	6-month WT	6-month ADTg	12-month WT	12-month ADTg	24-month WT	24-month ADTg
<i>n</i>	37	24	4	11	22	54	16	25
AP amplitude (mV)	101.8 ± 0.60	100.93 ± 0.85	100.53 ± 2.35	101.27 ± 1.14	101.02 ± 1.26	101.76 ± 0.80	100.44 ± 1.52	100.51 ± 0.72
AP threshold (mV)	-53.17 ± 0.55	-52.96 ± 0.53	-53.38 ± 1.65	-53.97 ± 0.96	-54.40 ± 0.71	-53.61 ± 0.56	-55.33 ± 1.06	-53.35 ± 0.79
AP half-width (ms)	1.61 ± 0.053	1.54 ± 0.048	1.55 ± 0.044	1.48 ± 0.049	1.59 ± 0.06	1.55 ± 0.05	1.56 ± 0.047	1.58 ± 0.05
rheobase current (pA)	90.14 ± 4.27	83.33 ± 4.61	81.12 ± 11.14	95.09 ± 9.35	98.18 ± 6.57	105.0 ± 7.46	80.44 ± 6.81	83.20 ± 6.89