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Evidence for Alzheimer's disease-linked synapse loss and compensation in mouse and human hippocampal CA1 pyramidal neurons

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Abstract

Alzheimer's disease (AD) is associated with alterations in the distribution, number, and size of inputs to hippocampal neurons. Some of these changes are thought to be neurodegenerative, whereas others are conceptualized as compensatory, plasticity-like responses, wherein the remaining inputs reactively innervate vulnerable dendritic regions. Here, we provide evidence that the axospinous synapses of human AD cases and mice harboring AD-linked genetic mutations (the 5XFAD line) exhibit both, in the form of synapse loss and compensatory changes in the synapses that remain. Using array tomography, quantitative conventional electron microscopy, immunogold electron microscopy for AMPARs, and whole-cell patch-clamp physiology, we find that hippocampal CA1 pyramidal neurons in transgenic mice are host to an age-related synapse loss in their distal dendrites, and that the remaining synapses express more AMPA-type glutamate receptors. Moreover, the number of axonal boutons that synapse with multiple spines is significantly reduced in the transgenic mice. Through serial section electron microscopic analyses of human hippocampal tissue, we further show that putative compensatory changes in synapse strength are also detectable in axospinous synapses of proximal and distal dendrites in human AD cases, and that their multiple synapse boutons may be more powerful than those in non-cognitively impaired human cases. Such findings are consistent with the notion that the pathophysiology of AD is a multivariate product of both neurodegenerative and neuroplastic processes, which may produce adaptive and/or maladaptive responses in hippocampal synaptic strength and plasticity.

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized by progressive memory dysfunction and accumulation of amyloid plaques and neurofibrillary tangles in the brain (Querfurth and LaFerla, 2010; Reitz et al., 2011). In brain tissue, AD is

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also associated with changes in signaling cascades (Camandola and Mattson, 2011; Hyman and Yuan, 2012), gene expression (Cooper-Knock et al., 2012; De Jager and Bennett, 2013), reorganization of connectivity (Hyman et al., 1989; Mesulam et al., 2004; Rekart et al., 2004; Rodriguez et al., 2012), synapse loss (Terry et al., 1991; Scheff and Price, 2006; Serrano-Pozzo et al., 2011), synaptic dysfunction (Malinow, 2012; Mucke and Selkoe, 2012) and neuron loss (Morrison and Hof, 2002; Giannakopoulos et al., 2009).

Though many of these changes are widespread, some brain regions are more affected in AD than others (Sperling et al., 2010; Small et al., 2011). For example, the medial temporal lobe shows significant AD-linked neurodegeneration, including neuron loss in the entorhinal cortex (Gomez-Isla et al., 1996; Kordower et al., 2001) and the hippocampus (West et al., 1994; 2004), as well as downregulation of synapse-related gene expression (Berchtold et al., 2013) and a loss of synapses (Scheff et al., 2006; 2007) and synaptic protein (Counts et al., 2014). These AD-linked changes have led to its conceptualization as a “synaptic disease,” especially within the hippocampus (Ferreira and Klein, 2011; Koffie et al., 2011; Selkoe, 2011; Spires-Jones and Hyman, 2014).

Given the difficulty of studying synapses during AD pathogenesis, transgenic mice harboring AD-linked genetic mutations represent a powerful means for examining the impact of prolonged exposure to high amyloid loads on synapses. Hippocampal synapses and their function are indeed altered in several lines of AD transgenic (ADTg) mice (Ashe and Zahs, 2010; Selkoe, 2011; Mucke and Selkoe, 2012). Hippocampal synapses are heterogeneous (Nicholson and Geinisman, 2009), though, and represent intrinsic or extrinsic connections (Amaral and Lavenex, 2007; van Strien et al., 2009). This segregation of inputs is of particular significance in ADTg mice expressing mutated forms of the amyloid precursor protein (APP). In such mice, the axons of entorhinal cortex neurons are major sources of APP, the protein precursor of neurotoxic and synaptotoxic A β peptides and amyloid plaques. Indeed, disruptions of these perforant path axons is sufficient to significantly reduce amyloid burden in the hippocampus of mice expressing the Swedish mutation of the human APP gene (APP^{Swe}; Lazarov et al., 2002; Sheng et al., 2002).

The most distal dendrites of hippocampal CA1 pyramidal neurons comprise the stratum lacunosum-moleculare (SLM; Amaral and Lavenex, 2007), which receives innervation from layer 3 of the entorhinal cortex. More proximal regions of the apical dendrites comprise the stratum radiatum (SR), and harbor connections between hippocampal regions CA3 and CA1. One possibility is that the distal dendrites of mice overexpressing the APP^{Swe} gene are vulnerable to amyloid toxicity being anterogradely transported from entorhinal cortex neurons. Another possibility is that the relative isolation of synapses in SLM from the cellular housekeeping machinery of the soma renders them vulnerable to AD-linked biochemistry or chronological aging in ADTg mice. In either scenario, distal synapses in SLM would be expected to show evidence for deconstruction or loss before those in SR.

To test this hypothesis, we investigated the effects of prolonged exposure to amyloid on synapses within the SR and SLM layers of dorsal hippocampal CA1 pyramidal neurons in 5XFAD (5xADTg) mice, which express five AD-linked mutations including APP^{Swe} (Oakley et al., 2006), and show age-related hippocampus-dependent learning impairments

(Kimura and Ohno, 2009; Ohno, 2009). We also examined synapses in CA1 tissue from human AD cases and their age-matched controls. Using multiple techniques, we find that synaptopathogenesis in 5xADTg mice and AD cases is consistent with a mixture of AD-linked synapse loss and putative compensatory changes. Interestingly, such changes resemble alterations described previously in aged rodents and primates (Bloss et al., 2011; 2013; Dumitriu et al., 2010; Hara et al., 2011), wherein compensatory rearrangements may actually be maladaptive, driving impairments in neuronal computation and plasticity through an altered balance of synaptic weights and a reduced number of the smallest synapses (Morrison and Baxter, 2012; 2014).

Materials and Methods

Animals

Male 5xADTg mice harboring the human APP Swedish mutation (K670N, M671L), Florida mutation (I716V), and London mutation (V717I) and two human presenilin 1 mutations (M146L and L286V) under expression of the Thy-1 promoter (Oakley et al., 2006) were obtained from The Jackson Laboratory (Bar Harbor, ME). Male 5xADTg mice were bred with female B6SJL wildtype mice (The Jackson Laboratory; Bar Harbor ME), and their male heterozygous offspring and male wildtype (WT) littermates were used. All mice were maintained in a ventilated micro-isolator cage and housed in the Rush University Medical Center Comparative Research Center.

The study used a total of 39 male mice, including 19 5xADTg and 20 WT. For the conventional electron microscopy experiments, 6 male 12-month old ($n = 3$ 5xADTg and $n = 3$ WT littermates) mice were evaluated. The array tomography experiments include 1-month old 5xADTg mice ($n = 2$), 1-month old WT mouse ($n = 1$), 12-month old WT mice ($n = 3$), and aged 5xADTg mice (10, 11, and 13 months of age; $n = 3$). Immunogold localization experiments used 11-month old mice ($n = 2$ 5xADTg and $n = 3$ WT littermate mice). Whole-cell patch-clamp physiology experiments are based on 4 recordings from three 1-month old WT mice; 4 recordings from three 1-month old 5xADTg mice; 9 recordings from 7 aged (11–15 months old) WT mice; and 17 recordings from 6 aged (11–15 months old) 5xADTg mice.

Use of Laboratory Animals

All animal procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Rush University Medical Center Institutional Animal Care and Use Committee.

Human cases

Postmortem human brain tissue was collected from male individuals with either AD ($n = 3$), who were participants in the Rush Alzheimer's Disease Center's Religious Orders Study; or males with no cognitive impairment (NCI; $n = 2$) from the University of Kentucky's community-dwelling cohort from the Biologically Resilient Adults in Neurological Sciences program (Scheff et al., 2007). Tissues were collected with the approval of the Human Investigations Committees at Rush University Medical Center and the University of

Kentucky's College of Medicine. Subject demographic are provided in Table 1. The AD cases did not have any familial AD-linked mutations and are classified as "mild" AD based on McKhann et al. (1984).

Conventional electron microscopy

Unbiased stereological serial section electron microscopy combined with unbiased systematic random sampling was used to estimate total synapse number in the proximal and distal one-thirds of the stratum radiatum (pSR and dSR, respectively) and the stratum lacunosum-moleculare (SLM) of the dorsal half of hippocampal region CA1 as we described previously (Nicholson et al., 2006; Nicholson and Geinisman, 2009; Menon et al., 2013) based upon the protocol of Geinisman et al. (1996). Mice were anesthetized with 90 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine, and transcardially perfused first with 500 mL of a solution consisting of 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M sodium cacodylate (both from Electron Microscopy Sciences, Hatfield, PA, Cat. numbers 15700 and 16350, respectively) and then with 300 mL of a solution consisting of 2% paraformaldehyde, 2.5% glutaraldehyde fixative in 0.1M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA; Cat. 12300) with 0.02 mM CaCl_2 (Electron Microscopy Sciences, Hatfield, PA; Cat. 12340). The animals' heads were post-fixed in the concentrated fixative overnight at 4° C followed by brain removal and dissection of the entire hippocampus. The hippocampus was then straightened, mounted on the stage of a tissue chopper (Gaarskjaer, 1978) with cyanoacrylate (Electron Microscopy Sciences, Hatfield, PA; Cat. 72588), and sectioned exhaustively into transverse slabs, with the first cut being positioned randomly within the first, most septal 300 μm of the straightened hippocampus and subsequent sections being separated systematically by 300 μm . This procedure yielded 15–20 slabs per mouse, which were segregated into dorsal and ventral halves (7–10 slabs from each half). All slabs from the dorsal half of the hippocampus from each mouse were then rinsed in 0.12M phosphate buffer (PB), osmicated with 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA; Cat. 19190) in PB, rinsed in PB, dehydrated through increasing concentrations of ethanol, and embedded in Araldite 502 resin (Cat. 13900). Slabs comprising the dorsal half of each mouse's hippocampus were then cured at 60° C for 48 hours. All reagents were obtained from Electron Microscopy Sciences (Hatfield, PA). Once cured, the thickness of each slab was measured, and each slab was mounted on a stub and sectioned with a diamond knife (Histoknife; Diatome, Hatfield, PA) and ultramicrotome (Leica EM UC6; Leica Microsystems, Wetzlar, Germany) to obtain a 1- μm thick histological section that contained the entire septal face of CA1. Histological sections were then mounted and stained with toluidine blue, photographed, and imported in ImageJ (Rasband, 1997–2012). The areas of CA1 stratum radiatum (SR) and SLM were measured on the light micrographs of each histological section from each slab, and the volumes of SR and SLM were estimated as the product of the summed areas and the average slab thickness. From each mouse, 4 slabs were chosen systematic randomly (with a random initial selection followed systematically with every other slab) along the septo-temporal axis of the dorsal half of the hippocampus, and among these slabs, the edge of the micrograph acquisition fields were chosen systematic randomly along the CA2-subiculum axis, which then was made the long edge of a trimmed sliver. Using a diamond knife (Ultra 35°; Diatome, Hatfield, PA), serial ultrathin sections were cut from each sliver, mounted on carbon-coated,

formvar-coated nickel slotted grids, and counterstained with 5% aqueous uranyl acetate and Reynold's lead citrate. A grid from each sliver was then used for serial section electron microscopy ($n = 4$ grids per animal, corresponding to a grid from each systematic randomly chosen slab for each animal). Using the field delineator of the electron microscope (JEOL 100CX and JEOL 1440EX; JEOL, Peabody, MA), the lengths of SR and SLM along the somatodendritic axis were determined and segregated into thirds. Within the proximal and distal third of SR (pSR and dSR, respectively) and SLM, a random field was chosen, and then photographed at 8,000X in each serial section (range: 25–37 sections). Electron micrograph negatives of serial sections were then scanned on an Epson 10000XL photocopier, aligned, and analyzed in ImageJ using the physical disector method (Sterio, 1984) after superimposing an unbiased counting frame with inclusion/exclusion lines (mean area = $74.2 \mu\text{m}^2$). The volume of each disector was the product of the area of the unbiased counting frame, the number of disectors ($n = 18$), and section thickness (average = 64 nm, determined for each series using Small's method of minimal folds). Only axospinous synapses were quantified. Perforated synapses were defined as those postsynaptic densities (PSDs) with at least one discontinuity in its profile in serial sections. The PSD profiles of nonperforated synapses were continuous in every section. The number of synapses detected in the disector volume was used to estimate synaptic numerical density, which was averaged across each slab from each animal, and then multiplied by the volume of the pSR, dSR, or SLM of each animal to obtain the estimated total number of synapses in the respective region of the dorsal hippocampus. Estimates are based on analyses of 10,704 axospinous synapses.

Array tomography

Array tomography was used to determine the number and density of PSD-95 immunofluorescent puncta in the 3-dimensional tissue volume from hippocampal slices, as determined using the Watershed program (generously provided by Stephen J. Smith and Brad Busse, Stanford University). This approach has been described in detail previously (Micheva and Smith, 2007; Micheva et al., 2010). Briefly, hippocampal slices from WT and 5xADTg mice were prepared for patch-clamp physiology, followed by whole-cell patch-clamp recordings (described below), fixation in 4% paraformaldehyde (3–7 days at 4°C ; made from Cat. 15700; Electron Microscopy Sciences, Hatfield, PA), and then processing for array tomography (Micheva et al., 2010; Menon et al., 2013). Dialyzed biocytin from the whole-cell internal patch solution was tagged fluorescently with a 1:300 dilution of streptavidin-conjugated Alexa Fluor 488 (Cat. S-32354; Life Technologies, Grand Island, NY), then fixed and dehydrated with microwave processing (Pelco BioWave Pro; Ted Pella, Inc. Redding, CA), embedded in LR white (Cat. 02646-AB; SPI Supplies, West Chester, PA), and cured at 52°C for 20 hours. Arrays ranging from 90 to 160 serial sections were collected using an ultramicrotome and diamond knife (Histo Jumbo; Diatome, Hatfield, PA) at a thickness of 70 nm and mounted on gelatin-subbed, high-precision coverslips (Cat. CSHP-No1.5-24x60; Bioscience Tools, San Diego, CA). Coverslips were immunostained with anti-PSD-95 antibody (1:200, mouse; Cat. 3450; Cell Signaling, Beverly, MA) and secondary anti-mouse Alexa Fluor 594 (1:150; Cat. Ab150117; Life Technologies, Grand Island, NY) followed by mounting with SlowFade Gold Antifade with DAPI (Cat. S36938; Life Technologies, Grand Island, NY). Images were acquired with a Zeiss Axioimager.M2

system equipped with an AxioCam MRM digital camera and AxioVision software (Carl Zeiss MicroImaging, LLC, Thornwood, NY). First, mosaics of the entire array at 10x were obtained, followed by creation of position lists of the same location in consecutive sections using MosaicPlanner (generously provided by Stephen Smith's laboratory at Stanford University), which were then imported into AxioVision to image 1×5 mosaics using a 63x/1.4 NA Plan Apochromat objective lens. Images were processed in Fiji (Schindelin et al., 2012) with plugins provided by Stephen Smith's laboratory-- first with ZviUnpacker followed by MetaStitch, then rigid alignment under the DAPI channel with MultiStackReg (provided by Brad Busse, Stanford University). The middle 60 sections were taken from each stack and pSR, dSR, and SLM were cropped, segregated from each other, and finely aligned with rigid then affine alignment in MultiStackReg. Using the Watershed program, PSD-95 image stacks were passed through a read filter, followed by thresholding to remove background noise using the "default" algorithm in Fiji. Then, individual 3-dimensional puncta were identified with a local maximum filter to detect peaks in local fluorescence, and segregated/segmented from nearby puncta in the 3-dimensional tissue volume created from serial sections. That is, single-section puncta that overlap on serial sections were segmented based on a local maximum and identified as a 3-dimensional punctum, representing a putative synaptic location, given the enrichment of PSD-95 at synapses (Funke et al., 2005). Four regions of interest (ROI; 11×8.8 μm) were taken from each region, taking care not to include capillaries or nuclei (or amyloid plaques), and analyzed with the Watershed program to acquire estimates of total PSD-95 synapse number in the array tomography tissue volume. Synapse number was divided by ROI volume (the product of ROI area, number of sections, and section thickness) to determine synapse density. Total volume of pSR, dSR, and SLM was measured in the arrays, which allowed us to estimate number of PSD-95 puncta in the arrays as the product of density and total volume of the boxed, rectangular sampling volume. Array tomography experiments include 2 1-month old 5xADTg mice, 1 1-month old WT mouse, 3 12-month old WT mice, and 3 aged 5xADTg mice (10, 11, and 13 months of age). Young 5xADTg mice did not differ from their age-matched WT littermate, or older WT mice. We therefore pooled their data for statistical comparisons to the aged 5xADTg mice.

Immunogold electron microscopy

Ultrastructural localization and quantification of postsynaptic immunogold particles for AMPARs in pSR, dSR, and SLM of dorsal hippocampal CA1 was performed as described previously (Ganeshina et al., 2004; Nicholson et al., 2006; Nicholson and Geinisman, 2009; Menon et al., 2013). Preparation and selection of tissue regions followed the systematic random sampling procedures described above for conventional electron microscopy with the following modifications. Briefly, 5 male, 11-month old mice (2 5xADTg and 3 WT littermates) were anesthetized and perfused transcardially with ice-cold 4% paraformaldehyde/0.5% glutaraldehyde in 0.12M PB. Both paraformaldehyde (Cat. 15700) and glutaraldehyde (Cat. 16300) were purchased from Electron Microscopy Sciences, Hatfield, PA. As with preparation of tissue for conventional electron microscopy, the hippocampus was dissected, straightened, and exhaustively sectioned into 300 μm slabs separated from each other systematic randomly by 300 μm. The dorsal half of such slabs (7–10 slabs total for each mouse) were then set aside, of which 4 were selected systematic randomly, and divided along the CA2-subiculum axis into 3 slivers (~500 μm wide in the

CA2-subiculum axis). Slivers were then cryoprotected in escalating concentrations of glycerol (Cat. 16550; Electron Microscopy Sciences, Hatfield, PA), plunge-frozen in liquid propane (-184°C) using a Leica EM CPC (Leica Microsystems, Wetzlar, Germany), transferred to a Leica AFS freeze-substitution system (Leica Microsystems, Wetzlar, Germany), then stained with 1.5% uranyl acetate in Fluka methanol (Sigma, St. Louis, MO) at -90°C , infiltrated with Lowicryl HM20 resin (Cat. 14340; Electron Microscopy Sciences, Hatfield, PA) at progressively escalated temperatures, and cured with ultraviolet light at 0°C . For each mouse, one sliver from each slab ($n = 4$ slivers total for each mouse, corresponding to each slab chosen from the dorsal hippocampus) was then used to prepare arrays of 16–42 serial ultrathin (67 nm) sections, mounted on carbon-coated, formvar-coated, gold-gilded nickel slot grids (Cat. FF205-Au, Electron Microscopy Sciences, Hatfield, PA.). For post-embedding processing, mouse tissue was prepared separately for each animal. Following preparation of arrays for electron microscopy, grids were immunostained in cohorts, such that grids from 1 WT and 1 5xADTg mouse were processed simultaneously. Grids were etched briefly with sodium ethoxide, treated with 0.1% sodium borohydride (Cat. 16940-66-2), and 50 mM glycine (Cat. 410225) in 5 mM Tris (Cat. T4661) buffer containing 0.05% Triton X-100 (Cat. T8532; all from Sigma-Aldrich, St. Louis, MO) and 0.9% saline (TBST), then rinsed in TBST, blocked with 10% normal goat serum (NGS; Cat. 25570; Electron Microscopy Sciences, Hatfield, PA) in TBST, and then incubated in a cocktail of primary antibodies to AMPAR subunits from Millipore (Billerica, MA) comprised of anti-GluA1 (AB1504; 3 $\mu\text{g/ml}$), anti-GluA2 (AB1768; 1.5 $\mu\text{g/ml}$), anti-GluA2/3 (AB1506; 3 $\mu\text{g/ml}$), and anti-GluA4 (AB1508; 3 $\mu\text{g/ml}$) overnight at 4°C . After rinsing in ultrapure, de-ionized, glass-distilled water, grids were blocked with 1% NGS in TBST ($\text{pH} = 8.2$), then incubated in a 1:50 dilution of secondary antibodies conjugated to 10 nm immunogold particles (Cat. 15726; Ted Pella, Inc., Redding, CA) and 1% NGS in TBST ($\text{pH} = 8.2$). After rinsing in TBST and water, grids were dried and counterstained as described above. As with the conventional electron microscopy experiments, systematic randomly determined regions within pSR, dSR, and SLM were selected to obtain electron micrographs on a JEOL100CX electron microscope in serial sections at 8000X, which were then scanned on an Epson 10000XL photocopier, aligned into stacks, and analyzed in ImageJ. Estimates are based on analyses of 9,194 axospinous synapses.

Whole-cell patch-clamp physiology

Preparation of hippocampal tissue for patch-clamp physiology from dorsal hippocampal CA1 pyramidal neurons followed the approach described previously, which isolates dorsal hippocampus from ventral hippocampus (Dougherty et al., 2012; 2013). Briefly, following isoflurane anesthesia, mice were perfused transcardially with ice-cold, oxygenated, sucrose-based artificial cerebrospinal fluid (aCSF) containing (in mM; from ThermoFisher Scientific, Waltham, MA): 210 sucrose (Cat. S3-500), 1.25 NaH_2PO_4 (Cat. BP329-500), 25 NaHCO_3 (Cat. S233-500), 0.5 CaCl_2 (Cat. C77-500), 7 MgCl_2 (Cat. M35-500), 7 dextrose (Cat. BP350-500), 1.3 ascorbic acid (Cat. A61-25), 3 Na-pyruvate (Cat. O4376-100). Mice were then decapitated, the brain was removed, followed by a trimming cut to the temporocaudal portion of the cortex, mounted on the latter on an ice-cold cutting stage, and then dorsal hippocampus slices (300 μm) were obtained in ice-cold, oxygenated sucrose-based aCSF with a Microm 650V thermally controlled vibrating microtome (ThermoFisher

Scientific, Waltham, MA). The slices were allowed to recuperate for 20 minutes in 37° C aCSF, followed by maintenance at room temperature for at least 1 hour in aCSF, both of which were constantly bubbled with 95% O₂/5%CO₂, until used for recording. The aCSF composition was as follows (in mM; from ThermoFisher Scientific, Waltham, MA): 125 NaCl (Cat. S671-500), 2.5 KCl (Cat. NC0227451), 1.25 NaH₂PO₄ (Cat. BP329-500), 25 NaHCO₃ (Cat. S233-500), 2 CaCl₂ (Cat. C77-500), 2 MgCl₂ (Cat. M35-500), 10 dextrose (Cat. BP350-500), 1.3 ascorbic acid (Cat. A61-25), 3 Na pyruvate (Cat. O4376-100). Slices were visualized using a fixed-stage, upright microscope (AxioExaminer; Carl Zeiss MicroImaging, LLC, Thornwood, NY) equipped with differential interference contrast videomicroscopy (NC-70; DAGE-MTI, Michigan City, IN). Whole-cell current-clamp recordings were obtained from CA1 pyramidal neurons using a Multiclamp 700B amplifier coupled to pCLAMP 10 data acquisition software (Molecular Devices, Sunnyvale, CA). The recording chamber was continuously perfused with oxygenated aCSF with SR95531 (Sigma-Aldrich, St. Louis, MO; 4 μM) and CGP55845 (Sigma-Aldrich, St. Louis, MO; 1 μM), and held at 32–34° C using an in-line heater (TC324B; Harvard Apparatus, Hooliston, MA). Patch pipettes were pulled (P-97 Flaming/Brown; Sutter Instrument, Novato, CA) from 1.5 mm outer diameter filamented borosilicate pipettes (World Precision Instruments, Sarasota, FL) to a resistance of 3–5 MΩ. Patch pipettes were filled with an internal solution containing (in mM): 115 K-gluconate (Cat. 157194; MP Biomedicals, Santa Ana, CA), 20 KCl (Cat. P217-500; ThermoFisher Scientific, Waltham, MA), 10 Na₂-PCR (Cat. P7936-25G; Sigma-Aldrich, St. Louis, MO), 10 HEPES (Cat. H4034-25G; Sigma-Aldrich, St. Louis, MO), 2 MgATP (Cat. A9187-500MG; Sigma-Aldrich, St. Louis, MO), 0.3 NaGTP (Sigma-Aldrich, St. Louis, MO., Cat. G8877-100MG), and 0.5% biocytin (Sigma-Aldrich, St. Louis, MO., Cat. B4261-250MG), at a pH of 7.3 and osmolality of ~285 mOsm. Series resistance and capacitance compensation were continuously monitored, and experiments were terminated if the series resistance exceeded 30 MΩ. Voltage signals were sampled at 10 kHz, Bessel filtered, digitized with a Digidata 1440A interface (Molecular Devices, Sunnyvale, CA), and transferred to a computer using pClamp 10 software. Membrane potential was held at –67 mV. We did not correct for the theoretical liquid junction potential,

At least 5 minutes after breaking into whole-cell configuration, spontaneous excitatory postsynaptic potentials (sEPSPs) were sampled from 15–20 separate 800 msec epochs for each neuron, separated by 30 sec. A hyperpolarizing pulse (200 msec; –50 pA) was used to continuously monitor input resistance. Digital signals were then smoothed using box-car averaging in pClamp10, after which events exceeding 0.1 mV and matching a 20-msec sEPSP template were selected, and visually confirmed to be sEPSPs. Events with rise times exceeding 5 msec or decay times exceeding 60 msec were excluded. As found in rats, events with slower rise times had higher amplitudes (Magee and Cook, 2000), but there were no effects of genotype, so all events were pooled for statistical comparisons. All neurons were visually confirmed to be CA1 pyramidal neurons using fluorescence-tagging as described above for array tomography procedures.

Human electron microscopy

Hippocampal tissue from the AD and NCI cases used in this study was from a previous electron microscopy study (Scheff et al., 2007). Briefly, the entire left hippocampal formation was removed, hand cut in 5 mm coronal sections, and immersion-fixed for 24 hours in 4% paraformaldehyde/1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO). Fixed tissue slabs were sectioned coronally at 100 μ m on a vibratome (Vibratome Co., St. Louis, MO), and random sections were chosen and post-fixed in 1% osmium tetroxide (OsO_4), stained en bloc with 0.5% uranyl acetate, dehydrated in graded ethanol and propylene oxide, and then infiltrated with epoxy embedding resin (Embed 812; Electron Microscopy Sciences, Hatfield, PA), and flat embedded and cured in circular molds at 60°C for 24 hours. Brains were coded, and analysis was done blind with respect to subject groups. Analyses were constrained to hippocampal slabs that had morphological characteristics of posterior hippocampus (within the hippocampal tail/body), which is homologous to dorsal/septal hippocampus in rodents.

Hippocampal region CA1 was dissected and mounted such that serial sections for electron microscopy were obtained perpendicular to the initial vibratome cut after autopsy. Blocks were trimmed to contain the alveus, pyramidal cell layer (PCL), stratum radiatum (SR), and stratum lacunosum-moleculare (SLM). Semi-thin sections (500 nm) were obtained and stained with toluidine blue in order to accurately define subregions of the apical dendrites and aid in additional trimming. Blocks were further trimmed such that only the PCL, SR, and SLM remained. Ribbons of 20–25 ultrathin (overall average = 62 nm, as determined using Small's method of minimal folds) serial sections were obtained on an Ultramicrotome (Leica EM UC6; Leica Microsystems, Wetzlar, Germany) and mounted on carbon-coated, formvar-coated nickel slot grids such that regions containing distal SR and SLM were centered on the grid slot (Electron Microscopy Sciences, Hatfield, PA). Grids were then stained with 5% uranyl acetate and Reynold's lead citrate. Series of 20–25 electron micrographs were obtained in the distal SR and SLM at an initial magnification of 7,500x. dSR was operationally defined as the region between the last pyramidal cell in the PCL and the beginning of the band of myelinated fibers that demarcates the perforant path in SLM. Serial section images from at least two progressively more distal fields from each region were obtained, allowing us to use proximo-distal location as a nesting factor within region for statistical comparisons. After obtaining images in serial sections, a final electron micrograph was taken at low magnification (100x) to ensure images were taken in the correct dendritic subregion. Labeling and measurements of synapses were performed as previously described, in ImageJ (Nicholson et al., 2006; Nicholson and Geinisman 2009). Briefly, all axospinous synapses were labeled within an entire series of 20–25 serial sections. Synapses were defined by the presence of a post synaptic density (PSD) in association with the presynaptic element containing at least three synaptic vesicles. Synapses with no discontinuities in the PSD were labeled as nonperforated while synapses with at least one discontinuity in the PSD were labeled as perforated. Only synapses in which the entire PSD profile was contained within the series were labeled and subsequently measured using NIH ImageJ software. As in the mouse tissue, PSD area was estimated as the product of the summed profile lengths across serial sections and average section

thickness for each series. Estimates are based on analyses of 522 synapses from 2 NCI cases and 809 synapses from 3 AD cases (see also Table 1).

Data and statistical analyses—All experiments and analyses were performed blind with respect to genotype/diagnosis. Data were compared statistically using Statistica (StatSoft, Tulsa, OK) with a $p < 0.05$ required to be significantly different, and post hoc tests were made using *a priori* single-factor pairwise comparisons (e.g., dSR of WT versus dSR of 5xADTg, etc.) with Fisher's Least Significant Difference procedure at $p < 0.01$. Data are presented as individual animal means. Statistical tests included repeated measures analysis of variance (ANOVA), multivariate ANOVA, multivariate analysis of covariance using PSD area as the covariate, and a nested ANOVA with proximo-distal location as the nested factor for statistical comparisons in the human tissue experiments. To compare data distributions, we used Kolmogorov-Smirnov tests of independent samples.

Results

Age-related distal synapse loss in 5xADTg mice

Many regions of the medial temporal lobe in 5xADTg mice exhibit evidence for amyloid plaque deposition (Oakley et al., 2006), including the entorhinal cortex, dentate gyrus, hippocampal regions CA3 and CA1, and the subiculum (Figs. 1A,B). In dorsal CA1, many of the amyloid plaques are found in association with the basal dendrites of stratum oriens (Fig. 1B), with relative sparing of the apical dendritic regions in SR and SLM (Fig. 1B). The absence of amyloid plaques in these regions, however, does not necessarily indicate that synapses are normal, as the length of SLM (in the somatodendritic axis) appeared smaller in the 5xADTg mice (Fig. 1B).

To test the idea that this ostensible volumetric atrophy of SLM was associated with AD-linked synapse loss, and that the apparent absence of pathology in SR indicated relative sparing of synapses in this region, we combined systematic random sampling with serial section electron microscopy to obtain unbiased estimates of total axospinous synapse number in SR and SLM in the dorsal hippocampus of 12-month old male 5xADTg mice and their age-matched, WT littermates. We estimated synapse number in two progressively distal regions of SR (the proximal and distal one-third of SR; pSR and dSR, respectively), and in SLM, the most distal apical dendritic region. In support of our initial observation of reduced SLM size, there is significant volumetric atrophy of SLM in the 5xADTg mice compared to their WT littermates, whereas their SR volumes are the same (Fig. 1B,C; $F_{(1,4)} = 18.68$, $p = 0.01$; ANOVA, genotype X region interaction).

Perforated and nonperforated synapses, which are categorized based on the configuration of their postsynaptic densities (PSDs; Fig. 1D), differ substantially with regard to size, abundance, and glutamate receptor expression (Ganeshina et al., 2004; Nicholson et al., 2006; Nicholson and Geinisman, 2009; Menon et al., 2013), indicating that they may also perform different functions supporting neuronal computation. Interestingly, the numerical density of all axospinous synapses is significantly reduced in SLM of 5xADTg mice (Fig. 1E; $F_{(2,16)} = 13.59$, $p = 0.0004$; ANOVA, genotype X region interaction). The combined impact of reduced SLM volume and lower synaptic numerical density in the 5xADTg mice

is a significant (~50%) loss of perforated and nonperforated synapses in SLM ($F_{(2,16)} = 15.58$, $p = 0.0002$, ANOVA, genotype X region interaction; Fig. 1F). Importantly, neither synapse density nor synapse number in SR is significantly different between 5xADTg and WT mice (Fig. 1E,F). Individual mouse data are provided in Table 2.

We also used immunofluorescence array tomography (Micheva et al., 2010) for PSD-95, a PSD-bound protein that acts as a scaffold for structural and signaling molecules (Funke et al., 2005), to provide a complementary examination of synapses in young and aged 5xADTg mice (Fig. 2A,B). Consistent with the volumetric atrophy in SLM detected in the electron microscopy experiments (Fig. 1B,C), aged 5xADTg mice have a significantly reduced SLM sampling volume compared to both their young 5xADTg counterparts and young and aged WT mice (Fig. 2A,B). The latter three groups did not differ from each other, nor did the sampling volume of SR differ among any group (Fig. 2A,B). We therefore pooled the data from the 1-month old 5xADTg mice with the data from the 1-month and 12-month old WT mice for statistical comparisons to the aged 5xADTg mice, since our main question was whether aged 5xADTg show evidence for synapse loss relative to young 5xADTg and both young and old WT mice. This approach revealed a significant loss of SLM sampling volume in the latter (Fig. 2C; $F_{(1,14)} = 6.73$, $p = 0.02$; ANOVA, group X region interaction). There is also significantly lower PSD-95 immunostaining in the aged 5xADTg mice (Fig. 2D; $F_{(1,21)} = 8.16$, $p = 0.009$; ANOVA, main effect of group) compared to the pooled control group. The width (along the CA2-subiculum axis) and depth (i.e., number of sections and section thickness) of the sampling volume is identical in all groups, so the length (along the somato-dendritic axis) of the sampling volume is the principal determinant of the reduced SLM volume in aged 5xADTg mice (Fig. 2A,B). We therefore estimated total PSD-95 puncta number in the boxed rectangular sampling volume as the product of PSD-95 puncta density and the sampling volume of SR and SLM, which revealed that the aged 5xADTg mice have a significantly lower number of PSD-95 puncta (Fig. 2E,F; $F_{(1,21)} = 8.73$, $p = 0.008$; ANOVA, main effect of group), as compared to the young 5xADTg mice and both groups of WT mice.

Increased synaptic AMPAR expression in the distal dendrites of 5xADTg mice

We next used serial section post-embedding immunogold electron microscopy (Fig. 3A,B) in pSR, dSR, and SLM of dorsal hippocampal region CA1 to determine whether aged 5xADTg mice show evidence of AD-linked dysregulation of synaptic AMPARs, which are altered upon exposure to A β peptides (Kamenetz et al., 2003; Hsieh et al., 2006). Consistent with previous work examining dendritic spine morphology (Merino-Serrais et al., 2011; Spires-Jones and Knafo, 2012), nonperforated synapses lacking AMPAR immunoreactivity, AMPAR-immunoreactive nonperforated synapses, and perforated synapses throughout CA1 are smaller in 11-month old aged 5xADTg mice as compared to those of their age-matched WT littermates (Fig. 4A), though this difference is statistically significant only among perforated synapses ($F_{(1,9)} = 33.24$, $p = 0.0003$; repeated measures ANOVA, main effect of genotype; and $F_{(2,9)} = 4.60$, $p = 0.04$, genotype X synaptic subtype interaction).

Surprisingly, we found that both perforated and nonperforated synapses in SLM of 5xADTg mice express more AMPARs than those of their WT littermates, despite their smaller sizes

(Fig. 4B). This observation regarding SLM synapses was validated statistically among perforated synapses via a significant rightward shift in the distribution of synaptic AMPAR expression levels (Fig. 4C; Kolmogorov-Smirnov test, $p < 0.01$), and via multivariate analysis of covariance using PSD area as the covariate for both AMPAR particle number (Fig. 4D; $F_{(2,6329)} = 13.22$, $p = 0.000002$; genotype X subtype X region interaction) and particle density (Fig. 4E; $F_{(2,6329)} = 4.0$, $p = 0.02$; genotype X subtype X region interaction).

No such differences in synaptic AMPAR particle numbers were detected among nonperforated synapses (Fig. 4F,G), though the smaller size of nonperforated synapses in SLM of the 5xADTg mice resulted in higher average particles densities compared to those of their WT littermates (Fig. 4H; $F_{(2,6329)} = 4.0$, $p = 0.02$; genotype X subtype X region interaction). Average data from individual mice are provided in Table 3.

Basal synaptic strength is preserved in aged 5xADTg mice

Although electron microscopy does not provide functional information, our observations indicate that synapses throughout the proximal apical dendrites are similar in 5xADTg and WT mice, both in terms of number and AMPAR expression, the latter being a major determinant of synaptic strength. By contrast, our experiments suggest that the strength of many synapses in the most distal dendrites (i.e., SLM) is upregulated. Synaptic potentials from these distal synapses are filtered substantially by dendritic cable properties, though, such that most if not all are immeasurably small at the soma (Magee and Cook, 2000; Williams and Stuart, 2003; Golding et al., 2005; Nicholson et al., 2006; Menon et al., 2013). Thus, it is reasonable to assume that the vast majority of spontaneous excitatory postsynaptic potentials (EPSPs) measured at the soma derive from SR and the basal dendrites (stratum oriens).

To verify our electron microscopic observations that SR synapses are similar in 5xADTg and WT mice with a functional assay, we used whole-cell patch-clamp physiology to record spontaneous EPSPs in dorsal CA1 pyramidal neurons from young and aged male 5xADTg and WT mice (Fig. 5A,B). Consistent with the absence of age-related synaptopathology in SR of 5xADTg mice (Fig. 1–4), neither the average somatic EPSP amplitudes (Fig. 5C) nor their distributions (Fig. 5D) differ from those of their age-matched WT littermates. EPSP amplitudes, however, are slightly larger in the aged mice of both genotypes ($F_{(1,30)} = 6.20$, $p = 0.02$; ANOVA, main effect of age). Thus, the close concordance between our electron microscopy experiments in SR and the somatic patch-clamp recordings support the notion that AD-linked synaptic alterations are not present in SR at these ages, but instead are found principally among the distal synapses in SLM.

Loss of multiple synapse boutons in the distal dendrites of 5xADTg mice

Our patch-clamp and immunogold electron microscopy experiments indicate that basal synaptic strength throughout the proximal dendrites is unchanged in aged 5xADTg mice, yet younger mice (~6 months of age) of this same transgenic line have been reported to have reduced field EPSP (fEPSP) input-output curves in CA1 SR when recorded at room temperature (Kimura and Ohno, 2009; Kimuro et al., 2010; Crouzin et al., 2013; see also Fitzjohn et al., 2001; Oddo et al., 2003; Chang et al., 2006, and Ricoy et al., 2011). These

contradictory findings may be related to the possibility that extracellular synaptic stimulation, as used in the fEPSP recordings, activates fewer synapses simultaneously in the 5xADTg mice, thereby reducing voltage signals from a population of neurons. Multiple synapse boutons (MSBs; Fig. 6A–C) are single axonal varicosities that synapse with more than one dendritic spine, the vast majority of which are on different neurons (Sorra and Harris, 1993; Yankova et al., 2001; Nicholson and Geinisman, 2009). Therefore, MSBs provide a means of depolarizing multiple spines and multiple neurons simultaneously. Using serial section conventional electron microscopy in pSR, dSR, and SLM, we estimated the total number of MSBs comprised only of nonperforated synapses (NP-NP), those involving a mix of nonperforated and perforated synapses (P-NP), and those comprised of two or more perforated synapses (P-P). Our analyses revealed that 5xADTg mice and their WT littermates have similar numbers of MSBs in pSR, but that aged 5xADTg mice have significantly fewer MSBs in dSR and SLM (Fig. 6D; $F_{(2,48)} = 11.10$, $p = 0.0001$; ANOVA, genotype X region interaction).

Previously, we found that the AMPAR expression of synapses involved in NP-NP or P-NP MSBs is either very low or disparate, with one synapse having significantly higher AMPAR expression than the others (Nicholson and Geinisman, 2009). Synapses involved in a P-P MSB in general show high AMPAR expression, but are comparatively rare (Fig. 6D; Nicholson and Geinisman, 2009). To determine whether such a pattern is maintained or disrupted in the aged 5xADTg mice, we identified 358 MSBs in the tissue prepared for immunogold electron microscopy for AMPARs. Though MSB synapses in both WT and 5xADTg mice are similar regardless of their subtype (Fig. 7A–H), perforated synapses involved in either P-NP or P-P MSBs have significantly higher expression levels for AMPARs in SLM of 5xADTg mice as compared to those of the WT littermates (Fig. 7G,H; $F_{(1,294)} = 4.18$, $p = 0.04$; repeated measures ANOVA, synapse X genotype X region X MSB subtype interaction). Taken together, these results indicate that there are significantly fewer MSBs in the distal dendritic regions of 5xADTg mice and that the perforated synapses with upregulated AMPAR expression in SLM are involved in MSBs.

Axospinous synapses on CA1 dendrites are larger in human AD brain

Previous electron microscopy studies of human brain tissue did not segregate synapses on dendrites from those on spines, nor between the perforated and nonperforated subtypes, nor excitatory versus inhibitory synapses (Scheff and Price, 2006; Scheff et al., 2006; 2007). This is an important issue to resolve, as increases in size (and by extension strength) among these various synapse types would have different functional implications. Moreover, the increases in synaptic strength in the axospinous synapses in 5xADTg mice suggest that the increased size among synapses in human AD brain may involve synapses on the dendritic spines of CA1 pyramidal neurons.

To address this issue directly, we used serial section electron microscopy to measure the size of excitatory, axospinous synapses in clinically diagnosed male AD cases and age-matched, non-cognitively impaired (NCI) male counterparts. We determined synapse size in the SR and the SLM of hippocampal region CA1 (Fig. 8A). SR is host to synapse loss in AD cases (Scheff et al., 2007), and SLM dendrites in primates, as in rodents, receive inputs from the

entorhinal cortex (Amaral and Lavenex, 2007). This homology provides cross-species relevance to the comparison to the data from the 5xADTg mice. As in the mice, both perforated and nonperforated synapses are present in tissue from NCI and AD cases (Fig. 8B), though synapses generally are sparser in SLM regardless of diagnosis ($F_{(2,14)} = 4.05$, $p = 0.04$; Wilks-Lambda nested ANOVA, main effect of region; NCI SR: 0.75 ± 0.11 nonperforated and 0.14 ± 0.03 perforated synapses per μm^3 ; NCI SLM: 0.38 ± 0.11 nonperforated and $0.07 \pm .03$ perforated synapses per μm^3 ; AD SR: 0.59 ± 0.09 nonperforated and 0.09 ± 0.02 perforated synapses per μm^3 ; AD SLM: 0.35 ± 0.02 nonperforated and 0.09 ± 0.02 perforated synapses per μm^3). In addition, as in rats (Nicholson and Geinisman, 2009) and mice (Fig. 4A), perforated synapses are larger than nonperforated ones. Notably, axospinous synapses are significantly larger in SR and SLM of AD cases as compared to their NCI matched controls (Fig. 8C,D; $F_{(2,14)} = 4.08$, $p = 0.04$; Wilks-Lambda nested ANOVA, main effect of diagnosis). These data indicate that increased synapse size among the excitatory axospinous synapses of AD hippocampus contributes strongly to the previously reported AD-related increases in PSD apposition length in CA1 SR (Scheff et al., 2007).

MSB synapses are larger in human AD hippocampus

We identified 49 MSBs in the human tissue, of which 34 were the NP-NP subtype (Fig. 9A,B; 24 MSBs from SR, 10 MSBs from SLM). When the sizes of synapses involved in these NP-NP MSBs were compared, we found that the synapses involved in MSBs in the AD cases are significantly larger compared to NCI subjects (Fig. 9C,D; $F_{(1,32)} = 4.85$, $p = 0.03$; repeated measures ANOVA, main effect of diagnosis), but that the difference in size between the larger and smaller synapses were, on average, similar in AD and NCI tissue (Fig. 9E). These results are consistent with the idea that small synapses are lost in AD, even those that are typically involved in NP-NP MSBs.

Discussion

There remains considerable uncertainty with regard to the molecular footprints of AD-linked synaptopathology and its underlying substrates. Our primary objective here was to examine the impact of prolonged *in vivo* exposure to AD-linked molecular processing on hippocampal synapses in transgenic mice harboring AD-related genetic mutations and in brain tissue from human AD cases. In both populations, we find evidence that (i) synapse loss involves a reduced frequency of the smallest synapses, which have been postulated to be the most plastic (Bourne and Harris, 2007; Zito et al., 2009; Kasai et al., 2010); and (ii) regions that exhibit synapse loss show evidence for compensatory rearrangements in synaptic weights. Our experiments further indicate that loss of small synapses may induce circuit rigidity via a reduced number of MSBs that involve nonperforated synapses. Interestingly, many such MSBs, though fewer in number, may actually have stronger synapses in both 5xADTg mice and human AD cases. Finally, synaptic alterations among 5xADTg mice are most prevalent in SLM, the target region of perforant path axons from the entorhinal cortex, whereas synapses in AD cases are affected in both SLM and SR. Thus, AD-linked synaptic alterations involve a mixture of plasticity and neurodegeneration, with

the former likely compensating for the latter, but perhaps resulting in both adaptive and maladaptive changes to hippocampal circuits.

Synapse loss in 5xADTg mice

The conventional electron microscopy and array tomography experiments provide strong evidence for age-related, progressive synaptic and volumetric atrophy in SLM, which culminates in a ~50% loss of synapses in the 5xADTg line of ADTg mice. These same experiments also suggest that synapse loss may occur in SR of aged 5xADTg mice, but perhaps as isolated microregions associated with amyloid plaque deposition (Dong et al., 2007; Koffie et al., 2009). Plaque load in dorsal CA1 in these mice, however, is considerably lower than in the subiculum, dentate gyrus, and hippocampal region CA3 (Figure 1B; Oakley et al., 2006; Kimuro et al., 2010; Shao et al., 2011). Therefore, the impact of such limited regional synapse loss on the overall population of SR synapses throughout dorsal hippocampal CA1 may be small (see also West et al., 2009).

Support for such an idea is illustrated in Fig. 2, where the presence of an amyloid plaque in the pSR of two of the boxed rectangular sampling volumes results in a substantial reduction of PSD-95 immunostaining in this region in the 5xADTg mice. In dSR, within which there were no amyloid plaques, PSD-95 immunostaining was the same in aged 5xADTg mice and their controls. Of note, the array tomography experiments were performed on hippocampal slices from which the patch-clamp data were obtained. The major finding of the patch-clamp physiology experiments was that basal synaptic function throughout SR and stratum oriens is similar in older 5xADTg and WT mice, indicating that if plaque-associated synapse loss occurred in the recorded neurons, there was no detectable effect on the strength of the remaining synapses in the proximal dendrites.

Importantly, both electron microscopy and array tomography revealed reductions in synapse number within SLM, which lacks amyloid plaques. Moreover, it was only among SLM synapses that we found compensatory increases in AMPAR expression. Together, these results suggest that there are at least two forms of synaptopathology present in the 5xADTg mice: (i) a local, plaque-associated loss of synapses regardless of input source that is not associated with compensatory changes in synaptic weight; and (ii) a global loss of synapses in the target zone of perforant path axons from the entorhinal cortex that is associated with compensatory increases in synaptic strength.

Why synapse loss is selective for the most distal dendrites of CA1 pyramidal neurons in 5xADTg mice is unclear. There are at least two possible explanations. First, expression of the APP^{swe} mutation in 5xADTg mice (Oakley et al., 2006) may generate amyloid toxicity via anterograde delivery from neurons in the entorhinal cortex. In another AD transgenic mouse that also expresses the familial AD-linked APP^{swe} mutation, disconnection of the entorhinal cortex from the hippocampus was sufficient to reduce amyloid plaque load in the dentate gyrus and CA fields (Lazarov et al., 2002; Sheng et al., 2002), indicating the perforant path axons are a major source of amyloid in the hippocampus. The most distal dendrites of CA1 pyramidal neurons comprise the SLM, which receives major afferent input from layer 3 neurons of the entorhinal cortex (Amaral and Lavenex, 2007). It is tempting to speculate that the synapse loss in the SLM of aged 5xADTg mice is driven by the long-term

exposure to secreted amyloid transported from the entorhinal cortex via axons of the perforant path. However, synapse loss may also reflect synaptic pruning due to ongoing reorganization of cortical and/or hippocampal microcircuits in mice expressing mutant forms of APP (Born et al., 2014) that are unrelated to AD per se, or from mutations in the presenilin-1 gene. Second, synapses on the distal dendrites can be located nearly 1-mm away from the cellular machinery of the soma. The reliance on housekeeping proteins synthesized in the soma, which clear away cellular debris and regulate important signaling proteins, could place such distal synapses at risk in aging, particularly in mice that express five mutant human transgenes. Indeed, neurons from human AD cases and from mice that model the disease show evidence for endosomal/lysosomal pathway dysfunction (Nixon, 2013), accumulations of autophagosomes (Kandalepas et al., 2013), and disruptions in postsynaptic morphology (Scheibel et al., 1976; Scheibel and Tomiyasu, 1978; Buell and Coleman, 1979; Probst et al., 1983; Yamada et al., 1988; Spires et al., 2005; Grutzendler et al., 2007; Wu et al., 2010), which may have cumulative effects on protein delivery, recycling, and regulation. Regardless of the underlying causes for selective synapse vulnerability, we demonstrate that it is age-related, AD-linked, and specific to the SLM. Since these alterations are a product of both synapse loss and volumetric atrophy, more work is needed to determine the constellation of substrates driving them.

The evidence for synapse loss we report here is specific to the distal dendrites. A previous examination of PSD95 immunofluorescence in 5xADTg mice also showed that signal intensity is decreased in the apical dendrites by 6-months of age (Shao et al., 2011), though these investigators find that PSD95 immunosignal is also lower in SR. Our electron microscopic experiments failed to find evidence for synapse loss in SR (Fig. 1), but there was some evidence for reduced PSD95 immunofluorescence when probed with immunofluorescence array tomography (Fig. 2; though such reductions appeared to be only in the neuropil near amyloid plaques). It is possible that experimental differences with regard to tissue preparation could explain the discrepancy, as our tissue was prepared either via perfusion with aldehydes (electron microscopy) or from slices used for patch-clamp physiology (array tomography), whereas Shao et al. (2011) used immersion fixation. Despite these differences, the present study and that of Shao et al. (2011) show that synapse loss is an emergent component of the synaptopathology of aging 5xADTg mice.

It is important to point out that we examined synapses in the dorsal half of the hippocampus from each mouse. The dorsal and ventral halves of the hippocampus, however, show differential plaque deposition in mouse models of AD (Oh et al., 2010; our unpublished observations). Such differential vulnerability to AD-linked neurodegeneration is also present in the homologous region of the human hippocampus (anterior hippocampus; Jack et al., 1997; Apostolova et al. 2010). It is possible that AD-related molecular processing affects synapses differently in ventral hippocampus, as compared to what we report here in dorsal hippocampus. It is also possible that synapse loss in ventral hippocampus influenced our stereological estimates. For example, estimates obtained from slabs near the middle of the hippocampus may resemble ventral slabs more than dorsal slabs with regard to amyloid load. Such a mixture would drive the estimates of the dorsal half down, even though it was only the slabs closer to the ventral half that displayed evidence of synapse loss. We failed to find any evidence for such a possibility when we examined the synaptic numerical density

estimates from individual tissue slabs closer to the middle of the hippocampus as compared to those obtained closer to the septal pole. Rather, in agreement with the array tomography experiments, the reduced synaptic numerical density was present in estimates from all dorsal slices, regardless of their septotemporal position.

Loss of synaptic connectivity in 5xADTg mice

In addition to synapse loss, there is a loss of connectivity in the older 5xADTg mice, wherein the number of MSBs in dSR and SLM is significantly reduced as compared to their WT littermate controls. Many MSBs are capable of simultaneously activating multiple neurons since the voltage-dependent machinery of neurotransmitter release (Südhof and Rizo, 2011; Jahn and Fasshauer, 2012) at such multiple-release sites is likely to be activated simultaneously by the propagating axonal action potential. Thus, the loss of MSBs likely has an impact on circuit function: it decouples clusters of hippocampal CA1 pyramidal neurons from single neurons in both hippocampal region CA3 and layer 3 of the entorhinal cortex. This decoupling in the 5xADTg mice indicates that their axonal boutons are connected predominantly to only one postsynaptic dendritic spine, providing an explanation for how extracellular stimulation of synapses in SR of 5xADTg mice, which are equal in number and AMPAR expression to WT mice, could produce smaller fEPSPs than those in WT mice (Kimura and Ohno, 2009; Kimuro et al., 2010; Crouzin et al., 2013).

Loss of MSBs may also be indicative of reduced circuit plasticity. In many regions throughout the brain, MSBs proliferate under conditions that involve plasticity, including reinnervation after brain lesions (Steward et al., 1988; Hatton et al., 1990; Jones, 1999; Meshul et al., 2000), sensory deprivation (Friedlander et al., 1991), estrous cycling (Woolley et al., 1996; Yankova et al., 2001), slicing-induced recuperative synaptogenesis (Kirov et al., 1999), acquisition of new skills and/or memories (Jones et al., 1997; 1999; Federmeier et al., 2002; Geinisman et al., 2001), and neuronal plasticity (Toni et al., 1999; 2007; Medvedev et al., 2012). The loss of MSBs in 5xADTg mice, then, may indicate that their hippocampal networks are less able to change in response to experience (see also Hara et al., 2011). We previously surmised that the MSBs involving nonperforated synapses are particularly important for circuit plasticity, whereas those involving only perforated synapses may represent stable and strong connections (Nicholson and Geinisman, 2009; see also Harris, 1995; Woolley et al., 1996). It is of interest, then, given their impairments in synaptic and behavioral plasticity (Oakley et al., 2006; Kimura and Ohno, 2009; Kimuro et al., 2010; Crouzin et al., 2013), that the MSBs involving nonperforated synapses are the only ones that are reduced in number in the 5xADTg mice. In future experiments, it will be of interest to determine whether slicing-induced recuperative synaptogenesis (Kirov et al., 1999; 2004; Sorra et al., 2006) is different in WT and 5xADTg mice, given the loss of MSBs in perfusion-fixed hippocampus reported here.

Importantly, some of our observations are at variance with previous studies of AD-linked molecular processing on synaptic AMPARs and postsynaptic changes. Specifically, studies in culture (e.g., Kamenetz et al., 2003; Hsieh et al. 2006), and in an ADTg mouse line harboring knockin mutations of both APP (K670N/M671L, APP^{swe} mutation) and presenilin-1 (P264L; Chang et al., 2006) provide evidence for impaired postsynaptic

processing, particularly with regard to AMPAR expression and function. Indeed, using ADTg mice and an experimental approach similar to the present study, Chang et al. (2006) found that the amplitude of miniature excitatory postsynaptic currents was lower in their ADTg mice by 9–12 months of age. In addition, they performed pre-embedding silver-intensified immunogold electron microscopy for AMPARs, and detected a slight leftward shift in immunoreactivity among synapses. We also used high-resolution techniques to probe for postsynaptic changes, including whole-cell patch-clamp physiology for sEPSPs and serial section post-embedding immunogold electron microscopy for AMPARs. Yet, we did not detect any postsynaptic changes within CA1 stratum radiatum. Furthermore, we reveal previously unreported morphological evidence that can explain reduced input-output fEPSP curves with presynaptic changes (i.e., loss of MSBs), rather than changes in postsynaptic AMPARs. There is no clear explanation for the discrepancies between our results and those of Chang et al. (2006), particularly in light of the fact that both mouse lines express overlapping mutations (i.e., APPswe). It is, however, important to note that post-embedding electron microscopy is particularly well suited for proteomically probing the dense protein matrix of the PSD (Amiry-Moghaddam and Ottersen, 2013). Nevertheless, both our study and Chang et al. (2006) use complementary experimental approaches, even though the conclusions are different. Note, however, that the idea that synapses get weaker postsynaptically is also in contrast with the electron microscopy experiments on synapses from human AD cases (e.g, Figures 8 and 9; Scheff and Price, 2006; Scheff et al., 2006; 2007). Clearly, more work using high-resolution approaches needs to be performed on AD mouse models and tissue from human cases.

Synaptic alterations in AD hippocampus

Previous electron microscopic studies of human AD brain tissue have provided evidence that regions linked to synapse loss in AD also have bigger synapses (Scheff and Price, 2006; Scheff et al., 2006; 2007). The present study is the first (to our knowledge) to analyze axospinous synapses in the SR and SLM of hippocampal region CA1 in AD cases using serial section electron microscopy. We find that both perforated and nonperforated synapses are larger in AD hippocampus than in their age- and sex-matched NCI controls, which as in the 5xADTg mice, depletes CA1 pyramidal neurons of their smallest synapses. Moreover, we show that, on average, the synapses that connect with MSBs in AD hippocampus are larger than those in NCI hippocampus. A reasonable interpretation of this observation is that synapses are lost in SR of AD cases (Scheff et al., 2007), but the strength of many of the remaining ones is increased at both single synapse and multiple synapse boutons. Such a notion also provides a link to the immunogold electron microscopic experiments using 5xADTg mice, wherein the synapses involved in single-synapse boutons and MSBs had higher AMPAR expression levels than those of their WT littermates in SLM, particularly among perforated synapses.

Relationship to the pathophysiology of AD

Any animal model of a human condition is inherently limited, particularly those involving insertion of human genes into their genome. At the very least, the 5xADTg mouse line is useful for understanding how long-term exposure to elevated amyloid levels affects the brain. Importantly, however, many ADTg mice only survive to a maximum of ~1.5 to 2

years (Ashe and Zahs, 2010; Selkoe, 2011), precluding a true examination of age-related, AD-linked pathophysiology. In addition, with 5 mutated genes in the 5xADTg mice, it is difficult to determine which, if any, mutation is driving the synaptopathologies reported here. There is strong evidence, though, that proteolytic processing of APP is a principal factor driving neurodegeneration in these mice, as genetic deletion of BACE1 is neuroprotective against numerous pathologies and behavioral deficits (Ohno et al., 2004; 2007). Overall, however, the observation that synapses from human AD cases are larger in brain regions that show synapse loss provides a link to our experiments in the 5xADTg mice. Thus, such changes may indeed be associated with AD-linked compensatory synaptic regulation in both mice and humans. The ability to identify similar pathophysiological substrates (i.e., increased synapse strength in regions that experience AD-linked synapse loss) is particularly striking, given that the 5xADTg mice express mutations associated with autosomal dominant, familial AD (FAD), whereas the human tissue derives from cases that were diagnosed with non-FAD sporadic AD. However, only perforated synapses showed evidence for AD-linked compensation in the 5xADTg mice compared to both subtypes in the human AD cases. One possibility is that 5xADTg mice older than those used here (i.e., older than 13 months of age) would display compensatory increases in strength among nonperforated synapses, similar to that seen in the AD cases, due to the progressive nature of the pathophysiology associated with continued exposure to AD-linked molecular processing. Another possibility is that these changes, despite their ostensible similarity, are not related, but are driven by transgene expression in the mice and some unrelated set of molecular pathogens in the human AD cases. In either case, we feel these results highlight areas to explore experimentally in future work.

Conclusions

We show here that there is an AD-linked loss of MSBs and axospinous synapses, particularly the smallest ones, in mouse and human hippocampal CA1 pyramidal neurons, as found previously in other brain regions of aged rats (Bloss et al., 2011; 2013) and non-human primates (Dumitriu et al., 2010; Hara et al., 2011) with age-related plasticity impairments. We also show that many of the remaining synapses are stronger in 5xADTg mice, and presumably in AD hippocampus based on their larger sizes. Therefore, both the 5xADTg mouse experiments and the human AD experiments are consistent with the idea that compensatory increases in synaptic weight may partially offset AD-linked synapse loss, but in so doing may also disrupt neuronal computation and induce circuit rigidity through the loss of the smallest, most plastic, and motile synapses (Morrison and Baxter, 2012; 2014).

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Fig. 1.

Age-related loss of synapses in the most distal apical dendrites of 5xADTg mice. (a) Fluorescent thioflavin S staining in the ventral hippocampus of 11-month old 5xADTg mice shows that amyloid plaque deposits are present in the entorhinal cortex (Ento), the dentate gyrus (DG), and hippocampal regions CA1 and CA3. (b) Toluidine-blue stained histological thick sections (500 nm) from dorsal hippocampal slices prepared for conventional electron microscopy from a 12-month old wildtype mouse (WT, top) and an 12-month old 5xADTg littermate (ADTg, bottom). SR: stratum radiatum; SLM: stratum lacunosum-moleculare. Note amyloid plaques in the DG (red asterisks), but not in CA1. Scale bar = 250 μ m. (c) Total volumes for stratum radiatum (SR) and the stratum lacunosum-moleculare (SLM) from the WT (black) and 5xADTg (red) mice. * = $p < 0.05$. (d) Electron micrographs of two serial sections through a perforated (top) and a nonperforated (bottom) axospinous synapse, with their 3-dimensional reconstructions (right). Scale bar = 500 nm. at: axon terminal; sp: spine; asterisks indicate perforations in the postsynaptic density (PSD); arrowheads denote the borders of the PSD. (e) Synaptic numerical density of perforated (top) and nonperforated (bottom) synapses in WT and 5xADTg mice. p: proximal stratum radiatum; d: distal stratum radiatum; s: stratum lacunosum-moleculare. * = $p < 0.05$. (f) Estimated total number of perforated (left) and nonperforated (right) synapses in WT and 5xADTg mice. * = $p < 0.05$.

Fig. 2.

Age-related loss of PSD-95 puncta in the apical dendrites of 5xADTg mice. (a) Aggregate/summed immunofluorescence array tomographic images for PSD-95 in young 5xADTg and young and aged WT mice. (b) Same as (a), but for aged 5xADTg mice. Scale bar = 100 μm . (c) Volume of SR and SLM for array tomography experiments for the pooled control group (black) and aged 5xADTg mice (red). * = $p < 0.05$. (d) Average PSD-95 puncta density in the three CA1 apical dendritic regions. * = $p < 0.05$. (e) Number of PSD-95 puncta in the volume of SR and SLM. * = $p < 0.05$. (f) 3-dimensional renderings of PSD-95 puncta from the array tomography volumetric tissue stacks, demonstrating that the length, width, and depth of the volumetric stack are the same in the pooled control group (top) and aged 5xADTg mice (bottom) in SR, whereas the length for the SLM volume is shorter in the latter due to atrophy. Scale bar = 100 μm . Arrows denote the boundaries of SLM, bordered by the

SR on its proximal end and the hippocampal fissure on its distal end. PCL = pyramidal cell layer; SR = stratum radiatum; SLM = stratum lacunosum-moleculare; DG = dentate gyrus.

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Fig. 3.

Immunogold electron microscopy for AMPARs at axospinous synapses. (a) Immunogold electron micrographs of three serial sections through a perforated synapse between an axon terminal (at) and a dendritic spine (sp) at low (top) and high (bottom) magnification. (b) Electron micrographs of two serial sections through three nonperforated synapses at low (top) and high (bottom) magnification. Scale bar = 200 and 400 nm, respectively. Arrowheads in (a) and (b) delineate the borders of the postsynaptic density (PSD). At the bottom are the three-dimensional reconstructions of the perforated synapse shown in (a) and the nonperforated synapses shown in (b). Gray: spine; aqua: PSD; black spheres: immunogold particles for AMPARs. Cube = 200 nm³.

Fig. 4.

Increased AMPAR expression at axospinous synapses in the most distal dendrites of aged 5xADTg mice. (a) PSD sizes (area in μm^2) for AMPAR immunonegative nonperforated, AMPAR immunopositive nonperforated, and perforated synapses from WT (black) and 5xADTg (red) mice. * = $p < 0.05$. (b) Scatterplots of immunogold particle number for AMPARs (on the ordinate) and PSD area (on the abscissa) for perforated and nonperforated synapses in SR and SLM of WT (black) and 5xADTg (red) mice. Lines denote the linear relationship, with all correlations at $p < 0.05$. (c) Cumulative frequency distributions for perforated synapses with a given number of immunogold particles from pSR, dSR, and SLM of WT (black) and 5xADTg (red) mice. Kolomogorov-Smirnov comparisons indicated that WT and 5xADTg synapses were different only in SLM (* = $p < 0.05$). (d) Immunogold particle number (per synapse) for perforated synapses in pSR, dSR, and SLM. * = $p < 0.05$. (e) Immunogold particle density (per μm^2 of PSD area) for perforated synapses. * = $p < 0.05$. (f) Cumulative frequency distributions for nonperforated synapses with a given number of immunogold particles from pSR, dSR, and SLM of WT (black) and 5xADTg (red) mice. Kolomogorov-Smirnov comparisons detected no differences. (g) Immunogold particle number (per synapse) for nonperforated synapses in pSR, dSR, and SLM. (h) Immunogold particle density (per μm^2 of PSD area) for nonperforated synapses. * = $p < 0.05$.

Fig. 5.

Somatic patch-clamp recordings show that spontaneous EPSP amplitudes in CA1 pyramidal neurons are similar in WT and 5xADTg mice. (a) Inverse monochrome images of dorsal CA1 pyramidal neurons tagged with streptavidin-conjugated Alexa Fluor 488 from an 11-month old WT (left) and a 13-month old 5xADTg (right) mouse. (b) Raw voltage traces from somatic patch-clamp recordings of WT (black) and 5xADTg (red) CA1 pyramidal neurons. Scale bars = 1 mV and 100 msec. (c) Spontaneous EPSP (sEPSP) amplitudes for young (left) and aged (right) WT and 5xADTg mice. * = $p < 0.05$. (d) Relative (bars) and cumulative (lines) frequency distributions for spontaneous EPSPs of a given amplitude in young (left) and aged (right) WT (black) and 5xADTg (red) mice.

Fig. 6.

Loss of multiple synapse boutons in 5xADTg mice. (a) Serial electron micrographs through a multiple synapse bouton (msb) involving only nonperforated synapses (sp1 and sp2; NP-NP). (b) Serial electron micrographs through a MSB involving a nonperforated (sp1) and a perforated (sp2) synapse (P-NP). (c) Serial electron micrographs through a MSB involving only perforated synapses (sp1 and sp2; P-P). Asterisks indicate the discontinuity, or perforation, in the postsynaptic density (PSD) of the perforated synapses. Arrowheads denote the borders of the PSDs. Scale bar = 400 nm. (d) Total number of MSBs in pSR, dSR, and SLM for WT (black) and 5xADTg mice (red). * = $p < 0.05$.

Fig. 7.

AMPA expression at multiple synapse bouton synapses. (a) A multiple synapse bouton (msb) involving two nonperforated synapses with very low AMPAR expression levels (sp1 and sp2). (b) A MSB involving two nonperforated synapses with disparate AMPAR expression levels (sp1 has no detectable AMPARs, whereas sp2 has five immunogold particles projected onto its postsynaptic density; PSD). (c) A MSB comprised of a perforated (sp1) and a nonperforated (sp2) synapse. (d) A MSB involving only perforated synapses (sp1 and sp2). Arrowheads denote the borders of the PSDs. Scale bar = 150 nm. (e,f) Immunogold particle number for the synapse with the higher (+) and lower (-) number of particles, or the perforated (P) and nonperforated (NP) synapses involved in MSBs with only nonperforated synapses (NP-NP), those with a mixture of perforated and nonperforated (P-NP), and those comprised of two or more perforated synapses (P-P) in WT (black, e) and 5xADTg (red, f) stratum radiatum. (g,h) Same as (e,f), but for MSBs in stratum lacunosum-moleculare.

Fig. 8.

Larger axospinous synapses in the apical dendrites of hippocampal region CA1 in Alzheimer's disease cases. (a) Light micrographs of a hippocampal slab obtained from a deceased 80-year old male with Alzheimer's disease. Red box on left is shown at a higher magnification on right. Blue micrograph is of a toluidine blue-stained histological thick (500 nm) section of the hippocampal slab after trimming it down for electron microscopy. Dashed blue line indicates the hippocampal fissure. DG: dentate gyrus; CA3: cornu ammonis 3; CA1: cornu ammonis 1; Sub: subiculum; PCL: pyramidal cell layer; SR: stratum radiatum; SLM: stratum lacunosum-moleculare; DG OML: dentate gyrus outer molecular layer. Scale bar = 325 μm (left) and 75 (right) μm . (b) Conventional electron micrographs of three serial sections through a perforated (top) and two nonperforated (bottom) synapses between axon terminals (at) and dendritic spines (sp) from human tissue. at: axon terminal; sp: spine. Scale bar = 500 nm. (c) PSD sizes (in μm^2) for perforated synapses in dSR and SLM in tissue obtained from non-cognitively impaired (NCI; black) and Alzheimer's disease (AD; red)

cases. * = $p < 0.05$. (d) PSD sizes (in μm^2) for nonperforated synapses in dSR and SLM of NCI (black) and AD (red) hippocampus. * = $p < 0.05$.

Fig. 9.

Synapses involved in multiple synapse boutons are larger in Alzheimer's disease cases. (a,b) Serial electron micrographs of two different multiple synapse boutons (msb) involving only nonperforated synapses (sp1 and sp2 in both a and b). Scale bar = 500 nm. (c,d) Individual (black, red circles) and average (white circles) postsynaptic density (PSD) sizes for the larger (+) and smaller (-) nonperforated synapse involved in individual MSBs in CA1 of (c) non-cognitively impaired cases (NCI) and (d) Alzheimer's disease cases (AD). * = $p < 0.05$. (e) The difference in size between the nonperforated synapses involved in individual MSBs from NCI tissue (black) and AD tissue (red).

Table 1

Subject profiles for AD and NCI cases

Age	Gender	Dx	MMSE	ApoE	PMI	Braak	CERAD	NIA Reagan	Institute
80	Male	NCI	26	E3E3	3	2	0	3	UK
80	Male	NCI	27	E3E4	3	4	0	0	UK
79	Male	AD	26	E4E4	4	3	2	2	RADC
80	Male	AD	15	E3E3	3	2	3	3	RADC
88	Male	AD	27	E3E3	4	5	2	2	RADC

Dx = Clinical diagnosis; MMSE = Mini-mental state examination; ApoE = Apolipoprotein E polymorphism; PMI = postmortem interval in hours; Braak = Braak stage; CERAD = Consortium to Establish a Registry for Alzheimer’s Disease score; NIA Reagan = National Institute on Aging-Reagan Institute score

Table 2

Synapse data from conventional electron microscopy experiments.

Animal	Volume ($\mu\text{m}^3 \times 10^6$)	Synaptic numerical density (per μm^3) [pSR, dSR, SLM]		Total number of synapses ($\times 10^6$) [pSR, dSR, SLM]		Number of synapses analyzed (pSR, dSR, SLM)	
		Wildtype	Perf	Wildtype	Perf	Wildtype	Perf
1	331.8 (SR)	1.7, 1.8, 0.91	0.16, 0.24, 0.42	204, 219, 102	18, 27, 69	515, 580, 163	52, 72, 110
	165.9 (SLM)						
2	357.9 (SR)	1.9, 1.8, 0.72	0.22, 0.39, 0.45	213, 198, 74	27, 46, 78	680, 663, 212	156, 208, 105
	175.0 (SLM)						
3	407.8 (SR)	2.3, 2.2, 1.0	0.25, 0.45, 0.37	276, 270, 111	34, 61, 75	700, 1092, 185	53, 90, 111
	202.6 (SLM)						
1	360.1 (SR)	2.2, 1.9, 0.45	0.26, 0.24, 0.19	265, 226, 49	31, 29, 21	419, 729, 98	49, 85, 47
	112.2 (SLM)						
2	331.7 (SR)	2.0, 1.7, 0.36	0.20, 0.33, 0.17	218, 186, 37	22, 37, 17	627, 576, 113	63, 113, 84
	102.7 (SLM)						
3	364.1 (SR)	2.3, 2.2, 0.35	0.25, 0.45, 0.26	276, 270, 39	31, 55, 28	756, 739, 141	84, 150, 84
	110.8 (SLM)						

Table 3

Synapse data from immunogold electron microscopy experiments.

Animal	PSD area (μm^2) [NP-, NP+, P]	Particle number (per synapse) [pSR, dSR, SLM]		Particle density (per μm^2 PSD area) [pSR, dSR, SLM]		Number of synapses analyzed (pSR, dSR, SLM)	
		Wildtype		Wildtype		Wildtype	
1 pSR	0.03, 0.04, 0.06	3.8, 4.2, 3.0	9.1, 10.3, 4.5	101, 121, 69	161, 169, 54	477, 991, 133	32, 65, 48
	dSR						
	SLM						
2 pSR	0.03, 0.04, 0.07	3.5, 3.6, 2.5	7.9, 8.8, 3.6	87, 87, 59	105, 152, 51	588, 962, 283	66, 83, 109
	dSR						
	SLM						
3 pSR	0.03, 0.04, 0.08	3.9, 4.2, 3.0	10.6, 11.5, 6.2	101, 102, 73	142, 167, 78	1096, 1200, 452	84, 103, 56
	dSR						
	SLM						
5xADTg	0.03, 0.04, 0.05	3.6, 4.2, 3.1	8.6, 10.0, 8.7	126, 100, 83	154, 130, 139	507, 409, 240	15, 28, 16
	dSR						
	SLM						
2 pSR	0.03, 0.03, 0.06	3.3, 3.3, 3.2	8.6, 12.9, 10.1	110, 112, 103	163, 210, 154	386, 430, 291	9, 15, 20
	dSR						
	SLM						

Animal	PSD area (μm^2) [NP-, NP+, P]	Particle number (per synapse) [pSR, dSR, SLM]	Particle density (per μm^2 PSD area) [pSR, dSR, SLM]	Number of synapses analyzed (pSR, dSR, SLM)			
	Wildtype	Nonperf	Wildtype	Nonperf	Wildtype	Nonperf	Perf
SLM	0.03, 0.03, 0.07						

Table 4
Synapse data from conventional electron microscopy experiments on tissue from human cases.

Dx	Age	Synaptic numerical density (per μm^3) [dSR, SLM]		PSD area (μm^2) [dSR, SLM]		Number of synapses analyzed (dSR, SLM)	
		<i>Nonperf</i>	<i>Perf</i>	<i>Nonperf</i>	<i>Perf</i>	<i>Nonperf</i>	<i>Perf</i>
NCI	80	0.71, 0.12	0.20, 0.05	0.03, 0.02	0.10, 0.05	117, 34	33, 13
NCI	80	0.79, 0.63	0.08, 0.08	0.03, 0.04	0.08, 0.08	135, 156	14, 20
		<i>Nonperf</i>	<i>Perf</i>	<i>Nonperf</i>	<i>Perf</i>	<i>Nonperf</i>	<i>Perf</i>
AD	79	0.52, 0.30	0.09, 0.05	0.05, 0.05	0.12, 0.14	196, 103	33, 18
AD	80	0.37, 0.42	0.06, 0.09	0.03, 0.03	0.05, 0.08	51, 82	8, 17
AD	88	0.80, 0.33	0.13, 0.13	0.05, 0.05	0.12, 0.09	209, 43	33, 17