HMW-AβOs in the induction and progression of synaptic or neuronal degeneration and provide a potential explanation for the extracellular one as the primary molecular basis for a toxic process.

Results

Generation of Aβ oligomer-specific monoclonal antibodies

Since the removal of AβMs is critical for the preparation of antigens to obtain AβO-specific antibodies, we isolated SDS-stable Aβ tetramers alone without any contamination of Aβ trimers and AβMs by SDS-PAGE (Fig. 1A). After *in vivo* immunization with the gel containing the Aβ tetramer alone, positive hybridoma supernatants were screened by dot blot analysis. Among positive supernatants (16/400, positive % = 4 %), two clones, namely, 1A9 and 2C3, were generated from a mouse that produced IgG2b. As shown by dot blot analysis, both 1A9 and 2C3 recognized soluble AβOs (100,000 g supernatant (sup) of 4-h-incubated mixture, Figs. 1B and 1C), not AβMs (560,000 g sup of seed-free preparation, Fig. 1B) or Aβ fibrils (100,000 g pellet of 120-h-incubated mixture, Fig. 1C), in contrast with 4G8 (Figs. 1B and 1C). The generation of A11- or 2C3-immunoreactive oligomers preceded that of 1A9-immunoreactive oligomers (Fig. 1B). The 1A9 conformer displayed a different

pattern of its time course, showing the highest stability in the oligomeric assembly state (Fig. 1B).

We further assessed the precise size of 1A9- or 2C3-immunoreactive AβOs. Blue native polyacrylamide gel electrophoresis (BN-PAGE), a charge shift method of electrophoresis, was carried out to determine the molecular mass and native oligomeric status of the 4-h-incubated mixture. As shown in Fig. 1D, the native Aβ species exhibit Aβ monomers, low-molecular-weight (LMW)-oligomers (dimers, trimers, tetramers, pentamers, and heptamers), and a high-molecular-weight (HMW)-oligomeric smear with molecular masses ranging in sizes from 66 to 720 kDa (Fig. 1D). Immunoblot analysis employing monoclonal 1A9 revealed its immunoreactivity with Aβ species corresponding to HMW-oligomeric smear of 480 to 1048 kDa (Fig. 1E), whereas 2C3 immunoreacted with Aβ species corresponding to a broad smear of 146 to 1048 kDa plus minute amounts of pentamers (Fig. 1E). The anti-oligo A11 immunoreacted with native Aβ species corresponding to a HMW-smear ranging in sizes from 242 to 1000 kDa (Fig. 1E), whereas monoclonal 4G8 detected Aβ oligomers larger than tetramers (Fig. 1E). The combination of two-dimensional native/SDS-PAGE and 4G8-immunoblot analysis revealed that 2% SDS disaggregates HMW-AβOs down to monomers and LMW-oligomers in addition to SDS-stable HMW-oligomers with

molecular masses corresponding to those of 8~40-mers (Fig. 1F). These findings suggest that the assignment of oligomer size is dependent on the method of evaluation. Indeed, monoclonal 4G8 detected all the Aβ species separated, whereas anti-oligo A11, 1A9, or 2C3 immunoreacted with SDS-stable 15-mers to 40-mers under our denaturing conditions tested (Fig. 1G), indicating that 1A9 or 2C3 is indeed specific to AβOs.

Characterization of neurotoxic AβOs

We further assessed the morphology of neurotoxic AβOs. After 0-h-, 2-h- or 4-h-incubation at 37℃, we tested the bioactivity of each incubated mixture (25 μM) by incubating NGF-differentiated PC12 (PC12N) cells [22] at 37℃ for 48 h. Surprisingly, Live/Dead two-color fluorescence assay [22] revealed that 2-h- or 4-h-preformed Aβ42 assembly decreased the toxic activity, whereas depletion of "insoluble" AβOs and concentration of "soluble" AβOs fully restored the toxic activity, similarly to that of Aβ42 assembly formed from seed-free fresh peptide (0 h) (Fig. 2A). These data indicated that seed-free fresh peptide (0 h) is the best source of *de novo* formation of neurotoxic AβOs. We isolated soluble Aβ species originated from seed-free fresh peptide by ultrafiltration and molecular sieving, which allowed us to separate the toxic Aβ42 peptide into five fractions (Fig. 2B). To verify the size distribution of toxic

Aβ42, PC12N cells were exposed to each fraction (25 μM) at 37℃ for 48 h (Fig. 2B). The level of LDH released from PC12N cells, when treated with each fraction, was similar to that treated with TBS alone. Live/Dead two-color fluorescence assay [22] revealed the toxicity of unfractionated Aβ42 or Frs. 3-5 (Fig. 2B, F (6/77) =39.85, $p<0.0001$), suggesting that toxic oligomers are at least larges trimers. Neurotoxic AβOs were then subjected to further dot blot immunoanalysis without SDS to determine their size, in which their "native" conformations are supposed to be maintained (Fig. 2C). Monoclonal 4G8 detected Aβ species in Frs. 2-5. The anti-oligomer A11 unequivocally reacted with toxic oligomeric conformers in Frs. 3-5. In contrast, 2C3 immunoreactivities were observed in Frs. 4 and 5, whereas minute amounts of 1A9 conformers were detected in toxic Fr. 5 alone. Taken together, these data suggest that both 1A9 and 2C3 toxic conformers are larger than 100 kDa and 30 kDa, respectively. Atomic force microscopy (AFM) [25] of toxic fractions revealed structures of different sizes and morphologies, including relatively compact spherical particles approximately 5-10 nm in diameter, large spherical particles roughly 25-50 nm in diameter, and an annular pore like structure 25 nm in inner and 75 nm in outer diameters (Fig. 2D).

1A9 and 2C3 immunoreactivitiy in human tissue

To investigate the distribution of 1A9 and 2C3 staining in human tissue, immunohistochemistry was performed on brain tissues obtained from 4 AD and 3 age-matched normal cases. The use of monoclonal 1A9 (Figs. 3A and 3I) and, to a lesser extent, monoclonal 2C3 (Figs. 3B and 3J) and polyclonal A11 (Figs. 3C and 3K) revealed that AβOs are highly localized within pyramidal neurons. Within these cells, AβOs accumulated as densely packed granules in the perikarya (Figs. 3A-C). Some of the diffuse plaques were stained by 1A9, but diffuse deposits were poorly stained by 2C3 and A11 (Figs. 3A-C). The majority of 1A9-stained pyramidal neurons exhibited atypical, eccentric large nuclei with abnormal chromatin morphology and distributions, features indicative of impending neuronal degeneration (Figs. 3A and 3I). Such abnormalities were less evident in 2C3- or A11-stained pyramidal neurons (Figs. 3B, 3C, 3J and 3K). Although age-matched control brains sometimes contained isolated clusters of AβO-burned neurons (Fig. 3D), the number of neurons involved and the amount of AβOs accumulated within these cells were much lower than those in AD brains (Figs.3D and 3I). Typically, 1A9-immunoreactive granules in control brains were relatively small, numerous, and rather uniformly distributed throughout the perikaryon of pyramidal neurons and the proximal portion of apical dendrites (Fig. 3D). In addition, intraneuronal AβOs were diffusely distributed throughout the perikaryon of

pyramidal neurons, which appears to be a characteristic feature in control brains (Figs. 3E and 3I-K). In AD brains, intraneuronal AβOs were sequestered into large, densely packed aggregates in the dendritic trunk and/or branches, and axons (Figs. 3A-C). Interestingly, some showed multiple dot-like accumulation of 1A9-immunoreactive AβOs arranged in tandem along apical dendritic shafts, which were focally swollen with an accumulation of 1A9-immunoreactive AβOs (Fig. 3F). The presence of intraneuronal Aβ was further supported by the staining by the widely used 4G8 (Fig. 3G) and 6E10 (Fig. 3H).

Extracellular AβOs are uptaken by neurons

To gain further insight into the link between extracellular and intraneuronal AβOs, neuroblastoma (SH-SY5Y) cells were incubated with 5μM HiLyte Fluor™ 488-labeled AβMs, AβOs, or Fluor™ 488 alone. Immunocytochemical analysis showed that fluorescence-labeled AβOs, not AβMs, bind to neuronal membrane for 10 min, followed by accumulation of intraneuronal AβOs for 0.5 or 3 hr (Fig. 4A). Vesicular uptake was not observed with fluorescence alone (Fig. 4A). Only when Fluor™ 488-labeled AβOs were added to SH-SY5Y cells, the LDH release assay [22] of cultures of SH-SY5Y cells revealed that the level of released LDH increased as a function of time

(Fig. 4B), suggesting that accumulation of intraneuronal AβOs induces neuronal cell death. The cell death was not detectable in the case of synthetic Aβ 42-1 and AβMs (Fig. 4B).

Monoclonal 1A9 or 2C3 immunotherapy protects Tg2576 from memory impairment

Using active 1A9 and 2C3 antibodies, we next evaluated whether a specific control of endogenous AβOs *in vivo* would be sufficient to prevent the disruption of neuronal function leading to memory loss. To assess this possibility, a long-term low-dose prophylactic study using 1A9 and 2C3 was designed instead of a therapeutic approach that was used previously by other scientists [26, 27]. Tg2576 mice were injected with 2C3 (n=12), 1A9 (n=13), or PBS (n=10) into the tail vein (0.4 mg/kg/week) from 4 months of age (that is, about 2 months before the onset of memory loss) until 13 months of age (when memory loss and amyloid plaque formation are already well established). Memory functions were measured in four behavioral paradigms, as described previously [28]: (1) short-term memory in the Y-maze test (Fig. 5A); (2) object recognition memory in a novel object recognition test (Fig. 5B); (3) spatial memory in the water maze test (Fig. 5C); and (4) associated emotional memory in the contextual fear learning test (Figs. 5D and 5E). Untreated Tg2576 mice (n=14) showed a significantly

poorer behavioral performance than untreated wild-type mice in all the behavioral paradigms tested (Figs. 5A-5E). Tg2576 mice treated with 1A9 and, to a lesser extent, 2C3 showed significantly better behavioral performance than untreated Tg2576 mice in all the behavioral paradigms tested (Figs. 5A-5E). Unlike that of untreated Tg2576 mice, the performance of 1A9- or 2C3-treated Tg2576 mice was indistinguishable from that of untreated wild-type mice (n=14) that had been previously tested in these tasks at this age [28], indicating that both short-term and long-term memories were well preserved in 1A9- or 2C3-treated Tg2576 mice.

Monoclonal 1A9 or 2C3 immunotherapy protects Tg2576 from synaptic degeneration and neuronal degeneration

The synaptoprotective effect was also confirmed at the postsynapse level, not at the presynapse level, at which the relative intensities of PSD-95 (Fig. 6A) to actin were significantly higher in 2C3-treated Tg2576 mice than in untreated Tg2576 mice. This is not the case with synaptophysin (Fig. 6A). Thus, 2C3 indeed protect Tg2576 mice from postsynaptic degeneration. In addition, the intensities of drebrin (Fig. 6A) relative to those of actin were significantly higher in 1A9-treated Tg2576 mice, indicating that 1A9 protects Tg2576 mice from the degeneration of dendritic spines.

This assumption was further supported by image analysis of synaptophysin, PSD-95, or drebrin after immunofluorescence microscopy analysis (Fig. 6A).

To further assess the neuroprotective effect of 1A9 or 2C3 immunotherapy, Fluoro-Jade B (FJB) binding assay, which specifically detects degenerative neurons, [29], was performed. As depicted in Fig. 6B, abundant FJB-positive neurodegenerative neurons were evident in untreated Tg2576 mouse brains. In contrast, such neurodegenerative neurons were negligible in 1A9- (Fig. 6B) or 2C3-treated Tg2576 mouse brains (Fig. 6B), indicating that 1A9 or 2C3 immunotherapy protects Tg2576 mice from neuronal degeneration.

Double-labeling analysis of AD brains (Fig. 6C) revealed that 1A9- or 2C3-positive neurons were degenerated as proven by Fluoro-Jade B(FJB) binding, indicating that the accumulation of intraneuronal AβOs is closely associated with neuronal degeneration (Fig. 6C).

Monoclonal 1A9 or 2C3 immunotherapy protects Tg2576 from the accumulation of AβOs

To gain further insight into the neurotoxic action of AβOs, we determined whether 1A9 and 2C3 immunotherapies targeting endogenous AβOs can prevent the

accumulation of extracellular or intracellular AβOs *in vivo*. A11-dot immunoblot analysis revealed that 2C3 immunization had a significant preventive effect ($p<0.05$ versus untreated group, post-hoc test) on the level of saline-soluble AβOs (Fig. 7A) which represents extracellular soluble AβOs. In contrast, 2C3 and 1A9 immunizations resulted in a significant reduction in the level of SDS-extractable AβOs (Fig. 7B), which represents mainly intracellular AβOs, owing to the specificity of 1A9 or 2C3 towards intracellular Aβ assembly (see Fig. 3). In the case of SDS-insoluble, FA-extractable fractions which represent highly insoluble AβOs, A11-dot immunoblot analysis showed that 2C3 immunization had a significant preventive effect ($p<0.001$ versus untreated group, post-hoc test) (Fig. 7C). In accordance with these data, double-labeling analysis revealed that 1A9 and 2C3 immunotherapies prevent the accumulation of A11-immunoreactive granules in the neurons regardless of the abundant A11-specific fluorescence found in untreated mice (Fig. 7D).

Discussion

The development of AβOs-selective antibodies has greatly facilitated the understanding of *in vivo* relevance of endogenous AβOs-mediated synaptic failure or neuronal degeneration. To prove this issue, a prophylactic study to control endogenous

AβOs using AβOs-selective antibodies is required. Several conformation-dependent antibodies such as oligomer- or fibril-specific antibodies were reported previously [12, 20, 21, 27, 30, 31], but none of them was examined for this purpose. In the current study, we successfully generated monoclonal oligomer-specific 1A9 and 2C3 using a novel design concept. Monoclonal 1A9 recognizes HMW-oligomers (100~230-mers), whereas 2C3 recognizes LMW- and HMW-oligomers ranging in sizes larger than pentamers (5~230-mers). In support of a previous report [12], prefibrillar oligomer-selective A11 appears to be specific to HMW-oligomers. Under conditions of SDS-PAGE, 1A9-, 2C3-, or A11-oligomeric conformers were consistently detected at 70-180 kDa corresponding to 15~40-mers. Note that neither 1A9 nor 2C3 reacted with monomers and fibrils. In spite of heterogeneity in size, AFM clearly demonstrated that the toxic 1A9-, 2C3-, or A11-oligomeric conformers display relatively compact spherical particles, not fibrilar structure.

Using these oligomer-selective 1A9 and 2C3, we found that the majority of AβOs exclusively accumulated in neurons, whereas the degree of staining of diffuse plaques varied among antibodies tested (1A9>2C3=A11). Under our conditions tested, A11-immunoreactivity is occasionally found in small diffuse plaques, in contrast with the finding of a previous report [12]. These findings are direct evidence that

heterogeneous oligomeric conformers exist as a distinct entity in both extracellular and intraneuronal deposits in human brains. Furthermore, the change of nuclear appearance in AβOs-burned neurons is highly indicative of impending neuronal degeneration: 1A9-positive AβOs may be associated with the most severe neuronal degeneration among the three anti-oligomer-specific antibodies tested. FJB binding assay, which specifically detects degernerating neurons [29], also confirmed that 1A9- or 2C3-burned neurons in AD brains were unequivocally FJB-positive, indicating that intraneuronal accumulation of AβOs is closely associated with neuronal degeneration.

Using above-mentioned 1A9 and 2C3, we evaluated whether a specific control of endogenous, extracellular AβOs *in vivo* would be sufficient to prevent the disruption of neuronal function leading to memory loss. Our *in vivo* investigation demonstrated that immunized subjects have less intraneuronal accumulation of AβOs and fewer degenerating neurons than untreated controls. In addition, 2C3 and 1A9 protected Tg2576 mice from postsynaptic degeneration and from the degeneration of dendritic spines, respectively. These results place both extracellular [22, 32] and intraneuronal AβOs [33, 34] centrally within the mechanisms mediating AβO-induced neuronal dysfunction leading to memory loss [26, 27]. In support of these data, our in vivo investigations clearly demonstrated that 1A9- and 2C3-treated Tg2576 mice aged 13

months showed cognitive performance superior to that of untreated Tg2576 mice, and, ultimately, performed better than and/or as well as untreated wild-type mice. It is unlikely that the impaired performance of Tg2576 mice in learning and memory tests is due to changes in motivation or sensory motor function, because the purpose of each behavioral test is different, and different skills are required for a good performance in each test. There were no differences in locomotor activity and the total time spent exploring objects in the novel object test between the wild-type and Tg2576 mice. Thus, it is likely that endogenous oligomeric 1A9- or 2C3-conformer is not only generated in Tg2576 mice, but is also actually a bioactive molecule *in vivo*, and its selective immunoneutralization by systemic administration of 1A9 or 2C3 is sufficient to prevent either short-term or long-term memory loss. This *in vivo* neuroprotective activity of 1A9 appeared to be superior to that of 2C3, supporting our current finding that neuronal degeneration in AD brains is more severe in 1A9-burned neurons than in 2C3-burned neurons. Recently, the generation of a new mouse model expressing only AβOs in neurons has demonstrated that endogenous AβOs are neurotoxic *in vivo* inducing synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss [35], supporting the pathological relevance of AβOs as shown herein. Taken together, the results from this study indicate that both extracellular and

intraneuronal AβOs represent a molecular basis of memory loss *in vivo*. Additional studies that attempt to identify the cellular and molecular targets on the cellular surface with which 1A9 or 2C3 interacts may yield insights into the mechanisms underlying the synaptotoxic or neurotoxic effects of AβOs or synaptoprotective or neuroprotective effect of 1A9 or 2C3.

Conclusions

We herein performed a hypothesis-driven, proof of concept study to prove the relevance of the *in vivo* Aβ oligomer hypothesis using monoclonal antibodies specific to AβOs generated using a novel design method. We found that AβOs are not only the real memory-relevant molecules, but also the real culprits of neuronal degeneration. Now, we have evidence that AβOs are among the earliest manifestation of the AD toxic process in mice and humans. We are certain that our studies move us closer to our goal of finding a therapeutic target and/or confirming the relevance of our therapeutic strategy.

Methods

Generation of monoclonal 1A9 and 2C3

Synthetic Aβ1-42 (r-peptide, Osaka, Japan) was dissolved in distilled, deionized H_2O, or 10 mM phosphate buffer at 250 μM, and allowed to incubate at 37°C for 18 h. Twenty microgram of preincubated Aβ1-42 was separated by NuPAGE 4-12% Bis-tris glycine gels, followed by CBB staining. Balb-c mice were immunized by injection with 2.5 μg of Aβ1-42 tetramer alone, which was excised from the gel and emulsified with complete Freund's adjuvant, into foot pads, followed by six additional injections. Inguinal lymphonode was used to generate hybridomas by fusion with Sp2/O-Ag14 myeloma cells with polyethylene glycol 1500. Initial screening was performed by dot blot analysis, applying 2.5 μl of seed-free fresh or 18-h preincubated Aβ1-42 (2.5 ng/dot) to a nitrocellulose membrane [22]. The blots were then allowed to dry and blocked with 5% low-fat milk and 1% BSA in PBS containing 0.05% Tween-20 (PBST) and incubated with culture medium supernatant, followed by horseradish peroxidase (HRP)-labeled goat anti-mouse or anti-rabbit $F(ab')_2$ antibody (1:3000; Amersham). Dot immunoblots were visualized with an ECL kit using LAS3000 mini (Fujitsu, Tokyo, Japan).

Preparation of Aβ1-42 peptide

Synthetic Aß1-42 was dissolved in 0.02% ammonia solution at 250 μM. To obtain

seed-free Aß42 solutions (540,000 g sup), the prepared solutions were centrifuged at 540,000 *g* for 3 h using an Optima TL ultracentrifuge (Beckman, USA) to remove undissolved peptides, which can act as preexisting seeds. The supernatant was collected and stored in aliquots at -80°C until use. Immediately before use, the aliquots were thawed and diluted with TBS (150 mM NaCl and 10 mM Tris-HCl, pH 7.4). For time-course experiments, 540,000 g sup was incubated for an indicated time (0-72 h), and soluble AßOs were retained after 100,000 g ultracentrifugation for 1 h, followed by dot immunoblot analysis (2.5 ng/dot) [22]. Because the 4-h-incubated mixture is suitable for the characterization of soluble AßO standards, we used it in further experiment instead of the 18-h-incubated mixture used in the first screening. To further assess whether monoclonal 1A9 or 2C3 recognizes Aß fibrils, seed-free Aß42 solutions (25 μM) were incubated for 5 days at room temperature. Electron-microscopy-confirmed Aß fibrils were subjected to dot immunoblot analysis (2.5 ng/dot) [22].

Electron microscopy (EM)

For electron microscopy, samples were diluted with distilled water and spread on carbon-coated grids. The grids were negatively stained with 1% phosphotungstic acid and examined under a Hitachi H-7000 electron microscope (Tokyo, Japan) with an

acceleration voltage of 77 kV.

BN-PAGE and two-dimensional Natie/SDS-PAGE

BN-PAGE analysis was performed following the manufacture's instruction (Invitrogen, Carlsbad, CA): 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, and 4-16% Novex® Bis-Tris gel was used. The apparent molecular masses of LMW-oligomers were calculated from the Ferguson plots with known molecular mass standards (α-lactalbumin, 14.2 kDa; carbonic anhydrase, 29 kDa; chickin egg albumin, 45 kDa; bovine serum albumin, 66 kDa monomer, and 132 kDa dimer; urease, 272 kDa monomer and 545 kDa dimer) (Sigma). For two-dimensional native/SDS-PAGE, one lane was excised from the gel and applied directly for SDS-PAGE. Immunoblot analysis was performed as described previously [36].

Aβ-induced toxicity assay

We conducted the Aß-induced toxicity assay according to previously published methods [22]. Briefly, rat pheochromocytoma 12 (PC12) cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated horse serum (Invitrogen) and 5% FBS (Invitrogen). For their differentiation, PC12 cells

were plated on 10-cm^2 poly-L-lysine-coated (10 mg/ml) dishes at a density of 20,000 cells/cm^2 and cultured for 6 days in DMEM supplemented with 100 ng/ml nerve growth factor (NGF; Alomone Labs, Jerusalem, Israel) (PC12N). Basically, toxicity was assessed using different Aß conformers: seed-free Aß1-42 vs. 2-h- or 4-h-preincubated Aß1-42 with or without 540,000 g ultracentrifugation for 1 h. PC12N was exposed to seed-free or preincubated Aß1-42 at 25 μM for 48 h at 4°C. Toxicity was assessed by Live/Dead two-color fluorescence assay (Molecular Probes, Eugene, OR) or CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, DI) in accordance with the manufacturer's instructions as described previously [22].

Ultrafiltration and molecular sieving (UF/MS)

To characterize the size of *de novo* formed toxic AβOs at 25 μM, serial ultrafiltration using Microcon® 3-, 10-, 30-, and 10-kDa cut-off membranes (Millipore Corp. Billerica, MA) was performed to prepare four filtrates (12,000 g-centrifuge for 90 min, Fr. 1, < 3 kDa; 12,000 g-centrifuge for 60 min, Fr. 2, 3-10 kDa; 12,000 g-centrifuge for 30 min, Fr. 3, 10-30 kDa; 12,000 g-centrifuge for 15 min, Fr. 4, 30-100 kDa) and one retentate (Fr. 5, >100 kDa). Each fraction at 25 μM was subjected to Aß–induced toxicity assay as described above. PC12N cells were exposed to each fraction and the toxic fraction

was assessed as described above. The distribution of the A11, 1A9, 2C3, or 4G8 conformer was characterized by dot blot analysis as described above. To determine the morphology of toxic oligomers, each fraction was also subjected to atomic force microscopy (AFM).

Atomic force microscopy (AFM)

AFM assessment was performed as reported previously [25]. Briefly, samples were dropped onto freshly cleaved mica. After allowing them to stand for 1 h, following by washing with water, the samples were assessed in a solution using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA, USA) set at tapping mode. OMCL-TR400PSA (Olympus, Japan) was used as a cantilever. Consecutive scans were monitored until distortion due to creep or shifts in the slow scan direction were negligible before collecting scans at sizes of 2 μm with the maximum 512 x 512 pixel resolution.

Immunohistochemistry

The left hemispheres of the brains of Tg2576 mice were sagitally cut into 30-μm-thick sections using a freezing microtome (RM 2145; Leika, Wetzlar, Germany). The tissue blocks from human subjects (4 AD subjects and 3 normal controls) or mice

were fixed in 4% paraformaldehyde with 0.1 M phosphate-buffered saline (pH 7.6) and embedded in paraffin wax. After deparaffinization, heat-induced antigen retrieval was achieved by boiling sections for 10 min in a microwave oven in 0.01M citrate buffer pH6.0, followed by 3 min incubation in 99% formic acid and then blocking of endogenous peroxidase. Then sections were subsequently incubated for 1 hour with primary antibody diluted in blocking buffer with normal goat or horse serum (2%), and after washing for 1 hour, with a secondary antibody in the same buffer. All incubations were done in parallel and photograph exposures were equal for sections in human and mice.

The following primary antibodies were used: monoclonal antibodies 6E10 and 4G8 against human Aβ sequence corresponding to residues 1-16 and 17-24, respectively, (Covance Immuno-Technologies, Dedham, MA), Polyclonal A11 specific to AβOs (Biosource, Camarillo, CA), anti-SYP (D4) antibody, monoclonal antibody against β-actin (C4) (Santa Cruz, Santa Cruz, CA), monoclonal anti-drebrin antibody (MBL, Nagoya, Japan), polyclonal anti-PSD-95 (CT) antibody (Invitrogen, Camarillo, CA), and our monoclonal 1A9 and 2C3 antibodies specific to AβOs.

The following secondary antibodies were used (1:1000): Goat anti-rabbit or anti-mouse IgG conjugated with horse-radish peroxidase (HRP) (Invitorogen, Carlsbad,