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,

CA), and Goat anti-mouse IgG conjugated with Alex Fluor (AF) 488 or 594 and goat anti-rat IgG conjugated with AF 488 (Molecular Probes, Eugene, OR). Immunopositive signals were visualized using an observation system (Compix Imaging System, Lake Oswego, OR) linked to an Olympus microscope BX50 through a highly sensitive CCD camera or a confocal laser scanning microscope (Carl Zeiss LSM510).

Fluoro-Jade B was purchased from Chemicon (now part of Millipore, Schwalbach, Germany). 7-AAD was purchased from Invitrogen (Carlsbad, CA).

Histochemistry

For Fluoro-Jade B histochemistry (1:50000), 5-μm-thick paraffin-embedded sections were deparafinized and stained following the manufacturer's instruction (Chemicon, now part of Millipore, Schwalbach, Germany). The Fluoro-Jade B-stained product fluoresces when exited at 488 nm and staining was imaged using a confocal laser scanning microscope (Carl Zeiss LSM510). The same procedure was applied for 30-μm-thick cryostat sections.

Cell culture and cellular uptake

Human neuroblastoma SH-SY5Y (SY5Y) cells were cultured in Dulbecco's modified

Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum. To investigate the fate of extracellular Aβ, SY5Y cells were exposed to HiLyte Fluor™ 488-labeled AβMs (10 kDa-filtrate), HiLyte Fluor™ 488-labeled AβOs (30 kDa-retentate) at 5 μM (AnaSpec, San Jose, CA) , or Fluor™ 488 alone for 10 min, 30 min, and 180 min. In a separate set of experiment, cultures were treated at 37°C for 0, 3, 6, and 24 h with 5 μM Fluor™ 488-labeled AβOs, and for 24 h with 5 μM Fluor™ 488-labeled AβMs and synthetic Aβ42-1 (AnaSpec, San Jose, CA). Toxicity was assessed by CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit in accordance with the manufacturer's instructions (Promega, Madison, DI) as described previously [22].

Protein extraction and immunoblotting

Saline-soluble, saline-insoluble, SDS-soluble fractions, or SDS-insoluble, formic acid (FA)-extractable fractions were prepared from the Tg2576 mouse brains as described previously [32]. Briefly, frozen brain samples were homogenized with a motor-driven Teflon/glass homogenizer (20 strokes) in TBS containing a cocktail of protease inhibitors (150 mg/ml), followed by centrifugation at 100,000 *g* for 1 h. The resultant supernatant (soluble fraction) was subjected to dot blot immunoanalysis or western blotting employing the same antibodies as those used for immunohistochemical staining.

The pellet was further extracted with 2% sodium dodecylsulphate (SDS), followed by 70% FA, and the homogenate was ultracentrifuged as described above. The resultant supernatant (insoluble fraction) was also subjected to dot blot immunoanalysis and western blot analysis. For western blotting, aliquots of isolated fractions were separated using NuPAGE 4-12% bis-tris-glycine gels and transblotted to nitrocellulose membrane or Immobilon P (Millipore) for 1 h at 400 mA using 10 mM 3-cyclohexylamino-1-propancsulphonic acid (pH 11) containing 10% methanol. Membranes were blocked for 3 h at room temperature with 5% low-fat milk and 1% BSA in PBST and incubated with either the polyclonal anti-A11 (1:1000) or anti-PSD95 antibody (1:250), and monoclonal anti-drebrin (1:100), anti-SYP (1:2000), and anti-β actin antibodies (1:1000), followed by HRP-labeled goat anti-rabbit or anti-mouse $F(ab')_2$ antibody (1:3000; Amersham). Immunoblots were visualized with an ECL kit using LAS3000 mini (Fujitsu, Tokyo, Japan). Densitometric analysis of immunoblot was performed using Multi Gauge v3.0 software (Fuji Film, Tokyo), and bands of interest were normalized to the corresponding actin bands indicated.

Immunization and behavioral analyses

All animal procedures were performed in accordance with a protocol approved by the

Animal Care Committee of the National Institute for Longevity Sciences. Several 3-month-old female nontransgenic (non-Tg) and Tg2576 mice that carry and overexpress the human APP gene with the Swedish double mutation (K670N; M671L) of familial AD were purchased from Taconics (Germantown, NY, USA) and maintained at our animal care facility until 13 months of age. To determine whether immunization prevents the development of Alzheimer-like phenotype, 4-month-old Tg2576 mice were administered 1A9 or 2C3 (0.4 mg/kg/week), or PBS intravenously via the tail vein until 13 months of age. Memory functions were measured at 13 months of age in the following four behavioral paradigms, as described previously [28]: (1) spontaneous alternation in the Y-maze test; (2) novel object recognition test; (3) Morris water maze test; and (4) cued and fear conditioning tests. Mice were sacrificed 3 days after the termination of the behavioral tests for biochemical and histological assessments. Experimental results were analyzed by one-way ANOVA and two-way ANOVA, with Fisher's test for post hoc analysis.

Spontaneous alternation in Y-maze test

The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converged at an

equilateral triangular central area that was 4 cm long at its longest axis. Each mouse was placed at the center of the apparatus, the sequence and number of arm entries were recorded for each mouse over an 8 min period. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternation behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two) multiplied by 100.

Novel object recognition test

The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was habituated to the box (30 x 30 x 35 cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed at the back corner of the box. A mouse was then placed midway at the front of the box and the total time spent exploring the two objects was recorded for 10 min. During the retention session, the mice were placed back in the same box 24 hr after the training session, in which one of the familiar objects used during the training was replaced with a novel object. The animals were then allowed to explore freely for 10 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects used were counterbalanced in terms of their

physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function. To eliminate the influence of the last behavioral test, the objects were changed each time.

Morris water maze test

The Morris water maze test was conducted in a circular pool (120 cm in diameter) filled with water at a temperature of 22 ± 1°C. A hidden platform (one block) (7 cm in diameter) was used. The mice underwent two trials (one block) for 10 consecutive days, during which the platform was left in the same position. The distance taken to locate the escape platform was determined in each trial using the Etho Vision system (Neuroscience Idea Co., Ltd., Osaka, Japan).

Cued and contextual fear conditioning tests

For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in the conditioning cage (17 x 27 x 12.5 cm) for 1 min, then in the conditioning cage (25 x 31 x 11 cm) for 2 min. For training (conditioning phase), mice

were placed in the conditioning cage, then a 15 sec tone (80 dB) was delivered as a conditioned stimulus. During the last 5 sec, an unconditioned stimulus was applied through a shock generator (Neuroscience Idea Co., Ltd.). This procedure was repeated 4 times at 15 sec intervals. Cued and contextual tests were carried out 1 day after the fear conditioning. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus. For the contextual test, mice were placed in the conditioning cage and the freezing response was measured for 2 min in the absence of the conditioned stimulus.

Ethics Statement

The research protocol was approved by the local animal esthetics committees at Research Institute, National Center for Geriatrics and Gerontology (Animal Care Committee) prior to initiation of the study. The research project was approved by the local ethics committee of Hirosaki University Graduate School of Medicine, and Research Institute, National Center for Geriatrics and Gerontology prior to initiation of the study.

Statistical analyses

We used factorial design analysis of variaence (ANOVA) or Mann-Whitney test to analyze data as appropriate. Significant ANOVA values were subsequently subjected to simple main effects analyses or post hoc comparisons of individual means using the Tukey's or Dannett's method as appropriate. We considered p values of 0.05 as significant for all studies. Some of the data obtained from animal experiments were analyzed by two-way ANOVA, with Fisher's test for post hoc analysis.

Competing Interests

YO, TY, and MS are the employees in Immunas Pharma Incorporation. TY and MS hold stock options in Immunas Pharma Incorporation. EM, TY, and MS are co-inventors of two filed provisional patent applications titled "Antibody Specific Binding to a Beta Oligomer and The Use" and "Antibodies That Specifically Bind to Aβ Oligomers and Uses Thereof" that cover the antibodies described in this paper, but this does not alter the adherence to all the Molecular Neurodegeneration policies on sharing data and materials. This study has in some parts been funded by a commercial funder, but that does not alter the author's adherence to all the Molecular Neurodegeneration policies on sharing data and materials.

Author's contributions

Conceived and designed the experiments: MM EM. Performed the experiments: AT YO TK AM TN HS TU NY EM. Analyzed the data: MS MS KY MM. Contributed reagents/ materisals/analysis tools: YO TY MS KA. Wrote the paper: AT EM. All authors read and approved the final manuscript.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Advanced Brain Scientific Project-from the Ministry of Education, Culture, Sports, Science and Technology, Japan [15016080 and 16015284 to E.M.]; and the Research Grant for Longevity Sciences from the Ministry of Health, Labour and Welfare [17A-1 to E.M.].

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Figure legends

Figure 1. Generation and characterization of oligomer-specific antibody. (A) Electrophoresis of immunogen. SDS-PAGE was performed to isolate the Aβ1-42 tetramer (red closed arrowhead) alone without any contamination by the Aβ1-42 trimer (black closed arrowhead) and Aβ1-42 monomer (opened arrowhead). Lane 1, Aβ1-42 dissolved in 10 mM phosphate buffer; lane 2, Aβ1-42 dissolved in distilled deionized water. (B) Aβ1-42 oligomer formation was observed as a function of time. Aβ1-42 monomer (25 μM) incubated at 37°C for the indicated time (0 – 72 h) were spotted on nitrocellulose membrane and subjected to a dot blot assay using A11 (1:100), 1A9 (1:50), 2C3 (1:50), or 4G8 (1:1000). (C) In this dot blot assay (left half of panel C), 1 μg of soluble Aβ42 oligomers (100,000 g sup for 4-h-incubation at 37°C) and Aβ42 fibrils (100,000 g pellets for 120-h-incubation at RT) were applied on a nitrocellulose membrane and probed with A11, 1A9, 2C3, or 4G8. EM image of fibrils (right half of panel C). (D) Characterization of Aβ1-42 oligomers under nondenaturing conditions. Aβ1-42 monomer (25 μM) incubated at 37°C for 4 h was separated on 16% BN-PAGE. (E) Separated peptides under nondenaturing conditions were also subjected to immunoblot analysis using A11, 1A9, 2C3, and 4G8. (F) The 100000 g sup of 4-h-incubated mixture of Aβ1-42 monomer (25 μM) was subjected to two-dimensional

native/SDS-PAGE, followed by 4G8-immunoblot analysis. SDS-stable 15~40-mers are indicated ( ] (red)). (G) Immunodetection of 4-h-incubated mixture of Aβ1-42 monomer (25 μM) under denaturing conditions probed with A11, 1A9, 2C3, and 4G8. SDS-stable 15~40-mers are indicated ( ] red).

Figure 2. Biophysical and structural characterization of neurotoxic Aβ assembly. (Upper half of panel A) Representative calcein AM/PI stainings of NGF-treated PC12 (PC12N) cells treated at 37℃ for 48 h with: TBS alone; 0-h preincubated Aβ1-42 (0 h); 2-h preincubated Aβ1-42 (2 h); 540,000 *g* supernatant obtained from 2 h (2 h sup); 4-h preincubated Aβ1-42 (4 h); 540,000 *g* supernatant obtained from 2 h (4 h sup). Green staining for viable cells versus red staining for dead cells. Resultant cell viability for each treatment is shown in lower half of panel A. Experimental results were analyzed by one-way ANOVA, followed by Tukey's test for posthoc analysis: statistical significance compared with TBS alone (*p<0.0001). Scale bar = 50 μm. (B) The seed-free Aβ1-42 (25 μM) was subjected to a series of membrane ultrafiltration steps with molecular cutoffs at 3, 10, 30, and 100 kDa. The resultant four filtrates and one retentate were designated as Fr. 1 (< 3 kDa), Fr. 2 (3-10 kDa), Fr. 3 (10-30 kDa), and Fr. 4 (30-100 kDa), and final retentate Fr. 5 (>100 kDa). The upper half of panel B shows