The neuroendocrine protein 7B2 suppresses the aggregation of neurodegenerative disease-related proteins*

Michael Helwig¹, Akina Hoshino¹, Casey Berridge², Sang-Nam Lee³, Nikolai Lorenzen⁴, Daniel E. Otzen⁴, Jason L. Eriksen², and Iris Lindberg¹

From the ¹Department of Anatomy and Neurobiology, University of Maryland - School of Medicine, Baltimore, MD 21201

²Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX 77204

³Research Center for Natural Human Defense System, Yonsei University College of Medicine, Seoul, Korea

⁴Department of Molecular Biology and Genetics, Interdisciplinary Nanoscience Centre, Aarhus University, 8000 Aarhus C, Denmark

*Running title: 7B2 inhibits protein aggregation

To whom correspondence should be addressed: Iris Lindberg, Ph.D., Department of Anatomy and Neurobiology, University of Maryland - School of Medicine, HSF II, Room S251, 20 Penn St, Baltimore, MD 21201, USA. Phone: 410-706-4778, Fax: 410-706-2512, Email: ilind001@umaryland.edu.

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Background: The neuroendocrine protein 7B2 blocks the aggregation of certain secreted proteins.


Conclusion: 7B2 inhibits the cytotoxicity of Aβ1-42 by modulation of oligomer formation.

Significance: 7B2 is a novel anti-aggregation secretory chaperone associated with neurodegenerative disease.

SUMMARY

Neurodegenerative diseases such as Alzheimer’s (AD) and Parkinson’s diseases (PD) are characterized by abnormal aggregation of misfolded β-sheet-rich proteins including beta amyloid-derived peptides (Aβ) and tau in AD, and α-synuclein in PD. Correct folding and assembly of these proteins is controlled by ubiquitously-expressed molecular chaperones; however, our understanding of neuron-specific chaperones and their involvement in the
pathogenesis of neurodegenerative diseases is limited. We here describe novel chaperone-like functions for the secretory protein 7B2, which is widely expressed in neuronal and endocrine tissues. In in vitro experiments 7B2 efficiently prevented fibrillation and formation of Aβ_{1-42}, Aβ_{1-40} and α-synuclein aggregates at a molar ratio of 1:10. In cell culture experiments, inclusion of recombinant 7B2, either in the medium of Neuro-2A cells or intracellularly via adenoviral 7B2 overexpression, blocked the neurocytotoxic effect of Aβ_{1-42} and significantly increased cell viability. Conversely, knock-down of 7B2 by RNAi increased Aβ_{1-42}-induced cytotoxicity. In the brains of APP/PS1 mice, a model of AD amyloidosis, immunoreactive 7B2 colocalized with aggregation-prone proteins and their respective aggregates. Furthermore, in the hippocampus and the substantia nigra of human AD- and PD-affected brains, 7B2 was highly colocalized with Aβ plaques and α-synuclein deposits, strongly suggesting physiological association. Our data provide insight into novel functions of 7B2 and establish this neural protein as an anti-aggregation chaperone associated with neurodegenerative disease.

INTRODUCTION

Excessive aggregation of misfolded proteins is a common feature in the pathophysiology of many neurodegenerative disorders, such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS) (1). Alzheimer’s disease, for example, is neuroanatomically characterized by extracellular plaques composed of amyloid precursor protein (APP)-derived amyloid beta (Aβ) peptides (2) and intracellular neurofibrillary tangles made up of hyperphosphorylated tau (3). Similarly, Lewy bodies, the hallmark of PD, are large cytosolic inclusion bodies composed of aggregated α-synuclein protein within dopaminergic neurons of the substantia nigra (4). Although the exact pathogenic role of these various aggregates is incompletely understood, it has been hypothesized that aggregation of Aβ peptides into oligomers and plaques results in a neurotoxic environment, disrupting cell function, and leading to the loss of specific neuronal populations (5).

In the search for underlying molecular mechanisms for these toxic effects, chaperone proteins have been implicated as important modulators of abnormal protein folding and aggregation in various neurodegenerative diseases (6). For example, several ubiquitously-expressed molecular chaperones within the heat shock (e.g. HSP90, HSP70 and HSP27) and α-crystallin protein families have been shown to be associated with protein misfolding diseases (7), (8), (9), (10). The secreted chaperone clusterin has also been implicated in neurodegenerative disease (reviewed in (11), (12)). However, our understanding of the role of chaperone-mediated quality control machinery in neurodegenerative disease is still limited, and the question of whether chaperones other than HSPs, crystallins, and clusterin might contribute to plaque pathogenesis or clearance remains open.

The secretory protein 7B2, known best for its role as a prohormone convertase 2 binding protein (proPC2) (13), (14), is universally expressed in endocrine, neural and neuroendocrine cells, which all possess a regulated secretory pathway (15), (16). Because expression of 7B2 in the brain is not confined to convertase-containing neurons (15), it seems likely that 7B2 must possess physiological functions exceeding its involvement in neuropeptide synthesis. Early reports indicated that 7B2 could be distantly related to a subclass of molecular chaperones called chaperonins (17). 7B2 blocks the formation of proPC2 oligomers and aggregates (18) as well as IGF-1 aggregates (19), demonstrating that 7B2 functions as a post-folding and post-secretion chaperone. Moreover, independent discovery studies searching for biomarkers of early-onset AD, PD, and ALS have identified 7B2 as a potential candidate protein (20); (21);(22); (23).
Based on findings showing association of 7B2 with neurodegenerative disease and the known role of 7B2 in blocking proPC2 aggregation, we have here investigated the hypothesis that neuronal 7B2 could function to block neurodegenerative disease-related protein aggregation. We tested the action of 7B2-derived proteins on the cytotoxicity and fibrillation of amyloid beta peptides Aβ1-42, Aβ1-40, and α-synuclein. Our experiments using animal, cellular, and in vitro approaches provide collective support for the idea that 7B2 represents a novel neuroprotective chaperone.

**EXPERIMENTAL PROCEDURES**

*Animal models*—All studies were conducted under University of Houston-approved IACUC protocols. 12-month old B6;Cg-Tg(APPswe,PSEN1dE9)85Db/J (APP/PSEN1; Jackson Laboratories) mice were used in this study. APP/PSEN1 double transgenic mice express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) protein, both directed to CNS neurons; these familial mutations are strongly associated with early-onset AD. Following sacrifice, brains were fixed with Accustain (Sigma Aldrich, St. Louis, MO) and subjected to paraffin processing.Brains were sectioned using a Leica microtome at 10 μm intervals.

**Immunohistochemistry of mouse brain tissue**—Ten μm coronal sections of formalin-fixed tissue were deparaffinized and subjected to an antigen retrieval protocol using Aqua DePar and Reveal antigen retrieval solutions in a Decloaking Chamber system (Biocare Medical). Following antigen retrieval, some sections were briefly stained with methoxy X04 (1 μM) followed by extensive washing to visualize dense core amyloid pathology. Other sections were treated with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), followed by treatment with 5% normal goat serum in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 20 min. Sections were incubated with polyclonal rabbit anti-7B2 (LSU13BF, 1:200) for one hour and washed with TBST. Sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories) for 30 min, washed with TBST, and then incubated with Texas Red Avidin DCS (Vector Laboratories) for 10 min. Sections were then washed with TBST. For co-localization, tissue was re-blocked using the avidin/biotin blocking kit, subjected to second round of blocking, and was then incubated with a second round of antibodies: anti-Aβ1-42 (1:250; 12F4, Covance), followed by washing and incubation with Fluorescein Avidin (Vector Laboratories) for 10 min. The sections were then washed extensively with Tris-HCl, mounted using Vectashield media and viewed under a confocal microscope (Olympus IX61 DSU). Images were processed with Neurolucida (MicroBrightField, Inc., Williston, VT).

*Human brain tissues*—An AD-affected human brain sample (female 73-year old donor) and a control sample (male 72-year old donor, naturally deceased) containing the hippocampus and a PD-affected human midbrain sample containing the substantia nigra of a male 89-year old donor were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. Formalin-fixed brain samples at either the level of the cortex or mesencephalon, respectively, were cryoprotected in 30% sucrose and deep-frozen in isopentane over dry ice (1 min) and stored at -80°C until required.

**Immunohistochemistry of human brain tissue**—Coronal sections (16 μm) containing the hippocampus (AD and control samples) and the substantia nigra (PD sample) were processed using a Leica cryostat, collected on Superfrost Plus object slides (Fisher Scientific, Hampton, NH) and treated with blocking solution containing 3% bovine serum albumin (BSA) in 0.5% Triton X-100 in PBS (0.5% PBS-T) for 1 h to block non-specific reactions. Sections were then incubated with polyclonal rabbit anti-7B2 antiserum (LSU13BF, 1:250) and monoclonal mouse anti-α-synuclein antiserum (1:150, BD
Biosciences, Franklin Lakes, NJ; (24) in blocking solution overnight (4 °C). Sections were rinsed briefly in 0.25% PBS-T and PBS and incubated with Cy3-conjugated (Ex<sub>max</sub> 550 nm, Em<sub>max</sub> 570 nm) goat anti-rabbit (1:200, A10520, Invitrogen, Carlsbad, CA) and Cy2-conjugated (Ex<sub>max</sub> 492 nm, Em<sub>max</sub> 510 nm) donkey anti-mouse (1:250, AP124J, Millipore, Billerica, MA) secondary antibody in blocking solution containing H33342 (Ex<sub>max</sub> 350 nm, Em<sub>max</sub> 461 nm) nuclear/DNA staining reagent (1:10,000, ALX-620-050, Axxora LLC, San Diego, CA) for 2h at RT. Sections were rinsed in PBS and coverslipped with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Immunofluorescence was visualized using a confocal Olympus BX61 (Olympus, Tokyo, Japan) and an epifluorescence Nikon Eclipse TE2000-E microscope (Nikon, Tokyo, Japan). Images of immunoreactivity were merged by color channel overlay using microscope-specific image processing software (Olympus FluoView, Nikon MetaView). Anatomical localization of immunoreactivity within the brain was annotated according to the Allen Human Brain Atlas and Gray’s Anatomy of the Human Body (30<sup>th</sup> Edition).

**Aβ<sub>1-42</sub> oligomer preparation**- Aβ<sub>1-42</sub> peptide films were resuspended in DMSO at a concentration of 5 mM and the peptide solutions were sonicated in a water bath sonicator for 10 min at room temperature. The solutions were then diluted to a final concentration of 100 μM with Ham’s F12 (phenol red-free, Biosource, CA) and were incubated at 4 C for 24 h to form Aβ<sub>1-42</sub> oligomers (25). In some experiments, Aβ<sub>1-42</sub> oligomers were added to the medium of Neuro-2A cells in the presence of either vehicle (Ham’s-F12); recombinant 7B2 (see below); α-crystallin (Sigma); or α-lactalbumin (Sigma L4385, 14.2 kDa).

**Cell proliferation and viability assay**- Neuro-2A cells were seeded at 5 x 10<sup>3</sup> /well in 96-well plates and left to attach at 37°C overnight. Subsequently, cells were treated with 10 μM Aβ<sub>1-42</sub> oligomers in the presence or absence of 2-4 μM 7B2, or α-lactalbumin as a negative control, or vehicle (Ham’s F12) for 48 h. Cell survival was measured at the indicated time points by adding 10 μl of a 1:3 (v/v) diluted WST-1 cell proliferation reagent stock solution (Roche, Mannheim). Samples were incubated for 60-240 min and absorbance at 450 nm was measured with a SpectraMax M2 fluorometer (Molecular Devices, Sunnyvale, CA, USA) using a 690 nm reference filter. After subtraction of the background absorbance, the mean values of the untreated control cells were set as 100%. In addition, cell viability was assessed by labeling cells with calcein AM (2 μM, Ex<sub>max</sub> 485 nm, Em<sub>max</sub> 530 nm, L3224, Invitrogen) and fluorescence was measured using a SpectraMax M2 fluorometer. Representative photomicrographs were taken using an epifluorescence Nikon Eclipse TE2000-E microscope.

**Adenoviral infection of Neuro-2A cells**- To infect 7B2-encoding adenovirus (26) into Neuro-2A cells, cells were seeded at 5 x 10<sup>3</sup> cells/well into 96-well plates. Replicate wells were trypsinized and counted again the following day for calculation of adenoviral multiplicity of infection (MOI). Cells were washed twice with PBS, and 7B2 or control (beta galactosidase-encoding) adenovirus was diluted to achieve an MOI of 1 in PBS in a final volume of 50 μl/well. The diluted adenovirus solution was added directly to cells in growth medium and the plates were swirled to mix well and incubated for 30 min to permit adenoviral infection. Fifty μl of high-glucose DMEM containing 2% fetal bovine serum were then added to each well. Adenovirus-infected cells were incubated for 36 h at 37 °C in a CO<sub>2</sub> incubator. The medium was then changed to DMEM containing 10 μM Aβ<sub>1-42</sub> for 48 h. Cell viability was assessed at this time using a WST-1 cell proliferation assay, as described above.

**7B2 RNAi experiments**- Three different specific sequences of stealth siRNA (Invitrogen, Carlsbad, CA) were designed for the murine 7B2 mRNA sequence (MSS237887, MSS237888, MSS237889). Following assessment of individual
knockdown efficiencies, the most effective siRNA, MSS237887, was deployed. A control scrambled sequence was designed to have the same GC content (46-2000; Invitrogen, Carlsbad, CA). Neuro-2A cells grown in 96-well plates were transfected sequentially with 100 nM of the respective siRNA on the first day and 200 nM on the second day using 5 µl/well of Lipofectamine 2000 (Invitrogen). The medium was then changed overnight to DMEM containing 4 µM Aβ1-42 for 48h. Transfection efficiency in Neuro-2A cells was monitored using a scrambled siRNA sequence conjugated to fluorescein (N2100S; New England Biolabs, Ipswich, MA). The total cell number was determined by counterstaining with 5 µg/ml Hoechst 33342 (Invitrogen) for 45 min and subsequent examination by fluorescence microscopy. Cell viability was assessed using the WST-1 cell proliferation assay, as described above.

Cellular uptake of 7B2 and Aβ1-42 into Neuro-2A cells- Neuro-2A cells were grown in 24-well plates on coverslips in DMEM supplemented with 10% fetal bovine serum (vol/vol) overnight. On the next day, 250 nM Fluor 647-labeled Aβ1-42 (Anaspec, Fremont, CA, Cat. 64161) and recombinant 7B2 was added to the medium and cells were incubated at 37 C, 5% CO2 for 24 h. Neuro-2A cells were then treated with 4% PFA for 20 min and incubated with blocking solution containing 3% bovine serum albumin (BSA) in 0.5 % Triton X-100 in PBS (0.5% PBS-T) for 1 h to block non-specific reactions. Exogenous 7B2 was then visualized using Alexa Fluor 488-labeled anti-His Tag antibody (Millipore, Billerica, MA; Cat 16-254). Cells were rinsed briefly in 0.25% PBS-T and PBS containing DAPI (Exmax 358 nm, Emmax 461 nm) nuclear/DNA stain reagent (1:10,000, ALX-620-050, Axxora LLC, San Diego, CA) for 2h at RT. Cells were then rinsed in PBS and mounted on object slides with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Immunofluorescence was visualized using a confocal Olympus BX61 (Olympus, Tokyo, Japan) and an epifluorescence Nikon Eclipse TE2000-E microscope (Nikon, Tokyo, Japan).

Peptide synthesis and purification of recombinant 7B2-derived peptides- An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the synthesis of the 7B2 86-121, a 36-residue peptide by the Fmoc procedure in NovaSyn TGR resin (Novabiochem, San Diego, CA) as described previously (27). The molecular weight and purity of the synthetic peptide were verified by reverse-phase HPLC and matrix-assisted laser desorption ionization-time of flight mass spectrometry (TofSpec E, Micromass). Recombinant His-tagged 27 kDa 7B2, 21 kDa 7B2, 7B2-30-150 and 7B2-68-150 were prepared using the QIAexpress system (Qiagen Inc., Chatsworth, CA). Primers were designed as described previously (27). PCR fragments were cloned into pQE30 and sequences were verified by DNA sequencing. Proteins were expressed in E. coli XL1-Blue (Stratagene, La Jolla, CA) and purified with the guanidine-HCl/refolding method, as previously described (28).

Thioflavin T assay- Fibrillation of amylogenic peptides (Aβ1-42, Aβ1-40 and α-synuclein) in the presence and absence of 7B2 was measured by thioflavin T (ThT) fluorescence assays in 96-well plates (29). Recombinant Aβ1-42 and Aβ1-40 were purchased from Biopeptide Co., Inc. (San Diego, CA) and α-synuclein was expressed and purified as described (30). Aβ1-40 and Aβ1-42 were diluted in 0.5% DMSO/0.5 M Tris-HCl buffer (pH 7.4, 1 mg/ml) and then diluted into 40 µM ThT solutions (in quadruplicate) containing or lacking the various forms of 7B2, in a total volume of 100 µl. α-synuclein fibrillation assays were performed in PBS (pH 7.4) and included one 3/32” diameter polytetrafluoroethylene bead (McMaster-Carr, Santa Fe Springs, CA) per well. The final concentrations of fibrillogenic peptides were 20 µM for Aβ1-40, Aβ1-42, and tau; and 44 µM for α-synuclein. Plates were incubated at 37°C with agitation on a microtiter plate shaker (Glas-Col, Terre Haute, IN), with the speed set to 30, for the time periods indicated. Controls for the fibrillation reactions included carbonic anhydrase (Sigma C5024, molecular
mass ~29 kDa), a protein chosen because of its comparable weight to 7B2. The development of fibrillation was monitored by measuring the fluorescence of ThT using a SpectraMax M2 fluorometer (Molecular Devices) at 485 nm emission (444 nm excitation).

**Dot blot analysis** - Fibrillated Aβ_{1-42} samples were centrifuged, transferred to a nitrocellulose membrane and subjected to anti-Aβ Western blot analysis. Aβ_{1-42} fibrillation assays were performed as described above. At 48h, samples of Aβ_{1-42} control reactions and of reactions incubated with 27 or 21 kDa 7B2 were removed. One-third of each reaction was used as a reference for the total reaction while the remaining material was centrifuged for 30 min at 20,000g (4 °C) to separate soluble material containing mono- and oligomeric Aβ_{1-42} and pelletable material containing heavy fibrils. An appropriate volume of PBS was added to total, supernatant and pellet samples to make the volume up to 100 μl. 10 μl of these reactions were transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA), air-dried, and the membrane then blocked with 0.5% BSA, 0.2% goat serum in Tris-buffered saline containing 0.3% Triton-X 100. The blot was incubated with monoclonal anti-beta amyloid antiserum (1:1000, 6E10, Covance, Princeton, NJ) in blocking buffer overnight at 4 °C. On the following day, the blots were washed three times with TBS containing 0.05% Tween, followed by incubation at room temperature for 1.5 h using horseradish peroxidase (HRP)- conjugate as secondary antibody. Blots were incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) for 1 min and chemiluminescent bands were visualized using HyBlot CL Autoradiography Film (Denville Scientific Inc.). Dot intensities were analyzed using ImageJ densitometric analysis software (NIH, Bethesda, MD), displayed as mean intensity (n = 3/group) and the ratios between supernatant and pellet samples were calculated.

**Transmission electron microscopy** - Specimens (incubated for 72 h) were adsorbed onto 400 mesh Formvar-coated copper grids and negatively stained with 2% phosphotungstic acid, pH 7. After wicking off excess solution, grids were air-dried and examined in a transmission electron microscope (Tecnai T12, FEI) operated at 80 kV. Digital images were acquired using an AMT bottom-mount CCD camera and AMT600 software.

**Luciferase refolding assay** - A possible ATP-dependent, chaperone-like protein refolding function of 7B2 was tested by incubating unfolded firefly luciferase with recombinant 7B2, followed by measurement of regained luciferase activity. A 10 μM solution of Photinus pyralis luciferase (Roche, Mannheim, Germany) was resuspended in 0.5 M Tris-acetate buffer (pH 7.5) followed by denaturation in 6 M guanidine hydrochloride and 5 mM DTT for 30 min at room temperature. The guanidine hydrochloride solution was dialyzed against PBS overnight at 4 °C. Refolding reactions were performed by diluting denatured luciferase 1:100 in refolding buffer (25 mM HEPES pH 7.4, 5 mM MgCl₂, 50 mM KCl, 5 mM DTT, 3 mM ATP) in the presence of recombinant 7B2 (4 μM) using BSA as a negative control and human recombinant HSP70 (Enzo Life Sciences, Farmingdale, NY) as a positive control. The incubation with 7B2 was performed for 3 h at room temperature, followed by 1:10 dilution of reactions into luciferase assay buffer (Promega, Madison, WI). Luciferase activity was determined by measurement of luciferin bioluminescence using a FlexStation3 microplate reader (Molecular Devices LLC, Sunnyvale, CA). Protein refolding was defined as recovery of luciferase activity, expressed as the percent of the activity of native luciferase measured at the same concentration.

**Statistical analysis** - Data were analyzed with one- or two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test, as appropriate, using a statistical software package (SigmaStat; Systat Software, Inc., San Jose, CA).
While α-synuclein colocalizes with Aβ1-42 amyloid plaque pathology in APP/PSEN1 mice —In order to assess a possibly physiologically relevant relationship between 7B2 and proteins involved in amyloid plaque pathology, we performed an immunohistochemical co-localization study using brains from 12-month-old APP mutant mice. Immunoreactive 7B2 was observed in association with Aβ1-42-immunoreactivity throughout the brain. Within the hippocampus, 7B2-immunoreactivity strongly overlapped with staining for Aβ1-42 (Fig. 1A). Furthermore, within the hippocampus, 7B2-immunoreactivity strongly colocalized with staining for Aβ1-42 dense core plaque pathology, as demonstrated by its apparent overlap with metoxy X04-positive Aβ1-42 aggregates (Fig. 1B).

7B2 colocalizes with Aβ1-42 deposits in human Alzheimer’s hippocampus and with α-synuclein-rich Lewy bodies within the substantia nigra of a Parkinson’s patient—7B2-immunoreactivity was detected throughout the extent of the human brain, including soma and dendritic and axonal branches in neurons of the cortex and mesencephalon (data not shown). Within the hippocampus of a human Alzheimer’s brain, 7B2 strongly colocalized with extracellular Aβ1-42 deposits (Fig. 2A). 7B2 strongly colocalized with α-synuclein-positive cytoplasmic inclusions (Lewy bodies) in neurons within the substantia nigra of a Parkinson’s disease brain (Fig. 2B). While over 80% of the observed 7B2 colocalized with α-synuclein-immunoreactivity, scattered α-synuclein-immunoreactivity could be observed which was not 7B2-immunoreactive (arrow with asterisk). In a similar hippocampus sample from a healthy human control, 7B2 staining was found near cell nuclei, indicating intracellular localization while no significant staining for Aβ1-42 could be observed, demonstrating no plaque pathology (Fig. 2C).

7B2 counteracts the neurocytotoxic effect of Aβ1-42 and increases cell viability—To investigate whether 7B2 is neuroprotective, we performed cell toxicity assays using Neuro-2A cells. A 48 h treatment of Neuro-2A cells with Aβ1-42 produced approximately 50% decrease in the number of living cells (Fig. 3A), as revealed by quantification of viable cells using both WST-1 assays (panel A, left) and calcein AM staining (panel A, right). Inclusion of 27 and 21 kDa 7Bs in the media of Neuro-2A cells during Aβ treatment significantly diminished Aβ-induced cell death. This effect was more pronounced when the 27 kDa form of 7B2 was added, and was dose-dependent. Aβ1-42-induced cell death was completely prevented when 27 kDa 7B2 was added into the medium together with Aβ1-42. Neither of the negative controls, carbonic anhydrase (a similarly-sized cytosolic protein) or α-lactalbumin (an irrelevant secreted protein) reduced Aβ neurotoxicity; however, α-crystallin, a known chaperone for Aβ (31), (32), was also able to block toxicity (results not shown).

In order to determine whether endogenously expressed 7B2 could also prevent Aβ neurotoxicity, we overexpressed 7B2 via adenoviral infection of Neuro-2A cells. A neuroprotective effect was observed when 7B2 was overexpressed by three-fold (Fig. 3B), resulting in a significant increase in living cells which reached ~85% percent of non-treated controls; these results indicate that endogenously-expressed 7B2 can rescue cells from Aβ1-42-induced neurotoxicity (Fig 3C). In a parallel experiment, we decreased intracellular 7B2 levels using 7B2-specific siRNA. The siRNA-induced decrease in 7B2 was accompanied by a similar decrease in the number of viable Neuro-2A cells; control oligonucleotides showed no such deleterious effect. We observed some colocalization of exogenously-added recombinant His-tagged 7B2 with fluor-labeled
Aβ1-42 within Neuro-2A cells, indicating cellular uptake of both proteins into the cytosol of Neuro-2A cells (Fig. 3D).

7B2 inhibits the fibrillation of Aβ1-42, Aβ1-40, and α-synuclein in vitro – In order to elucidate the molecular mode of action by which 7B2 inhibits the formation of cytotoxic Aβ species, and to obtain structure-function information, we performed in vitro fibrillation assays. The addition of full-length 27 kDa 7B2 (Fig. 4A and for a more detailed representation Fig. S1A) inhibited the fibrillation of Aβ1-42 at molar ratios of 1:10 (7B2 : Aβ1-42). Structure-function analysis using truncated forms of 7B2 revealed that this anti-aggregation effect is greatest when the protein is full-length (Fig. 4B) and moreover, is dose-dependent (Fig. 4C). In agreement with these findings, the majority of Aβ1-42 became insoluble during the course of the fibrillation assay, as shown by dot-blot analysis of centrifugally-separated Aβ1-42 fibrillation reaction samples which indicated greater formation of aggregates following 48 h of incubation (Fig. 4D). The ratio of soluble/lighter Aβ1-42 to insoluble/heavier Aβ1-42 oligomers (supernatant: pellet), however, shifted to favor soluble Aβ1-42 species when reactions were co-incubated with either 21 or 27 kDa 7B2, suggesting inhibition of the generation of larger fibrils by these proteins. Blockade of fibril formation was independently substantiated by quantification of transmission electron microscope images of Aβ1-42 fibrils and oligomers (Fig. 4E) incubated at 37 °C for 48 h, which demonstrated a substantial decrease in fibril length when 27 kDa 7B2 was added to the reaction. While fibrils in the non-treated Aβ1-42 samples reached an average length of 575 ± 302 nm (mean ± SD, n = 10) following incubation, fibril length decreased by about 80% when 7B2 was included in the reaction (to 124 ± 233 nm). In these samples, we also observed an increase in the number of smaller spherical Aβ1-42 aggregates with an average diameter of 10 ± 4 nm.

The addition of 7B2 to pre-incubated Aβ1-42 samples (at the time point indicated by an arrow) did not result in the disintegration of pre-formed Aβ1-42 fibrils, indicating that 7B2 does not function as a disaggregate (Fig. 5A). However, consistent with its effects on Aβ1-42 fibrillation, 7B2 also inhibited the formation of Aβ1-40 (Fig. 5B) and α-synuclein fibrils (Fig. 5C). A dose-dependent relationship was observed for blockade of α-synuclein fibrillation by 27 kDa 7B2 (Fig. 5D).

We next aimed to determine if 7B2 exhibits ATP-dependent chaperone-like refolding properties similar to those of larger chaperones such as members of the HSP70 and HSP90 families. While denatured and unfolded luciferase was efficiently refolded by HSP70, restoring about half of its enzymatic function, 7B2 displayed no significant refolding activity (Fig. S1B).

The small molecular mass of 7B2 potentially qualifies this protein to be classified as a small heat shock protein (sHSP); these chaperone proteins possess masses between 15 and 40 kDa and share the conserved sequence known as the α-crystallin domain (ACD (33)). However, multi-sequence alignment using 3DTCoffee Web server tools revealed no significant sequence identity with either α-crystallin itself or with the comparably-sized heat shock protein sHSP27 (Fig. S1C).

**DISCUSSION**

Neurodegenerative diseases such as AD and PD have been established as ‘protein folding disorders’ whose etiology involves the aggregation of non-native protein conformations, resulting in extracellular and intracellular protein aggregates (34). The abundance of these protein aggregates in neurodegenerative disease is, however, difficult to explain and appears to represent an essential failure of neuronal chaperone systems to
sustain native protein conformation. The particular toxicity of protein aggregates in the nervous system implies that neurons may require special mechanisms to maintain continuous chaperone control of protein aggregation during secretory pathway transit, granule residence, and even following secretion and reuptake, yet few specifically neuronal secretory chaperone mechanisms have been described.

Although 7B2 has long been recognized as an excellent neuroendocrine marker involved in PC2-mediated peptide synthesis (13) (14), its widespread neuronal distribution within the brain, also in areas lacking prohormone convertases (15), strongly suggests non-convertase-related functions. Our immunohistochemical data show clear colocalization of 7B2 with aggregated proteins in neurodegenerative disease, indicating a potential functional relationship. A similar accumulation of chaperones within amyloid-like plaques in the brains of AD and PD patients has been reported for α-crystallin, HSP47, and clusterin (8); (35); (36). Interestingly, the distribution of immunoreactive 7B2 in diseased brain indicates that this protein may possess a higher affinity for non-aggregated Aβ1-40 and Aβ1-42 and Aβ1-40, and a lesser affinity for fully mature dense core plaques. This suggests an anti-aggregation effect of 7B2 that is temporally organized, occurring prior to compaction of Aβ deposits as would be expected from a chaperone-mediated defense response to Aβ plaque maturation. A general association of 7B2 with neurodegenerative protein aggregation is further substantiated by the pronounced intracellular colocalization of 7B2 with α-synuclein-rich Lewy bodies within the substantia nigra of a PD patient.

7B2 is found together with intracellular as well as extracellular protein aggregates, implying that it could act at both locations. We speculate that 7B2 may block inappropriate protein-protein interactions initially during intracellular protein trafficking through the secretory pathway; extracellularly following secretion of 7B2 and Aβ; and possibly even following reuptake. Interestingly, cellular reuptake of Aβ into endosomes and lysosomes has been reported to facilitate its aggregation (37). Here we show that Neuro-2A cells are capable of taking up added Aβ simultaneously with added exogenous 7B2, raising the idea that these compartments serve as sites for 7B2 interaction with Aβ under physiological conditions. The cytoplasmic protein α-synuclein has been reported to be secreted and to be localized intravesicularly in a similar fashion (38); an analogous mechanism could be operative for α-synuclein/7B2. However, whether extracellular association of these proteins occurs prior to uptake, or whether both species are non-specifically endocytosed together, is not yet clear.

We further demonstrate here that added 7B2 can block the cytotoxic effects of Aβ peptides. Neuro-2A cells exposed to toxic Aβ oligomers die rapidly; inclusion of either 7B2 or alpha crystallin, but not the control proteins carbonic anhydrase or α-lactalbumin, blocked the cytotoxic effects of these oligomers. It is unlikely that fibril formation is involved in Aβ neurotoxicity, since the concentration of Aβ used was low (10 μM) and since Aβ oligomers appear to be much more toxic than fibrils (39). Thus, 7B2 may function to block protein-protein association at the oligomer level; this idea is in line with the known ability of 7B2 to block both the oligomerization as well as the aggregation of the convertase proPC2 (18).

Of great physiological importance is our observation that modulation of intracellular 7B2 expression directly correlates with the cytotoxicity of exogenously administered Aβ1-42. In a similar approach, Magrane et al. have shown that viral overexpression of HSP70 successfully rescues neurons from the toxic effects of intracellular Aβ accumulation (40). Moreover, another study demonstrates that drug-induced chaperone overexpression of HSP70 and HSP90 maintains tau protein in a
soluble and functional conformation, preventing it from aggregating (10). In line with these studies, our data indicate that a certain level of endogenous 7B2 may be sufficient to prevent the formation of harmful Aβ₁₋₄₂ species and supports the idea that loss of neuronal 7B2 might facilitate Aβ₁₋₄₂-induced neurotoxicity. The location of 7B2 action in blocking Aβ₁₋₄₂-induced neurotoxicity requires further investigation; it is possible that 7B2 acts both extracellularly to block oligomer formation, and intracellularly following reuptake.

In addition to these in vivo and cellular data linking 7B2 with the toxic effects of Aβ, we have directly demonstrated that 7B2 suppresses the fibrillation of aggregation-prone proteins in vitro. The ability of 7B2 to act at low stoichiometric ratios with respect to client proteins is remarkable, and further supports the idea of 7B2 involvement in Aβ-related plaque formation.

Most protein chaperones described to date function intracellularly. Only four secreted chaperones which act extracellularly have been identified thus far: (i) the ubiquitously-expressed glycoprotein clusterin/ApoJ, which in the brain appears to be of glial origin (11); (ii) the heat shock-related lens protein α-crystallin (41); (iii) the presumed beta amyloid chaperone prostaglandin D synthase/beta trace (42); and the receptor-associated protein RAP (43). Of these, the chaperone with by far the greatest genetic association with AD is clusterin (reviewed in (11), (12)). A key feature which distinguishes 7B2 from clusterin is that clusterin is expressed in nearly all mammalian tissues, while 7B2 expression is limited to cells containing a regulated secretory pathway: the central and peripheral nervous systems and endocrine/ neuroendocrine systems. Interestingly, a recent study investigating the interaction of clusterin and Aβ using biophysical approaches reported that clusterin binds to and stabilizes Aβ oligomers of all sizes, thereby influencing the equilibrium of Aβ oligomers, aggregates, and fibrils (44). It is possible that 7B2 acts via similar molecular mechanisms to decrease Aβ protein oligomerization, fibrillation and cytotoxicity. The appearance of the disc-shaped structures observed by EM in this study seem to represent an increase in spherical Aβ₁₋₄₂ oligomers, potentially indicating that 7B2 promotes the formation of non-toxic stable off-pathway Aβ species, similar to what has been reported following treatment with inositol (45).

Biophysical characterization of recombinant 7B2 has shown that it is an intrinsically disordered protein capable of oligomerization (46). These properties are similar to those of certain small anti-aggregant cytosolic heat shock proteins (shHSPs; reviewed in (47), (48)), and we speculate that 7B2 may block the formation of protein aggregates using similar mechanisms. While weak homology to chaperonin-related sequences has been reported (17), 7B2 exhibits no significant sequence homology with clusterin, crystallins, or shHSPs. Thus, 7B2 appears to have novel anti-aggregation domains. The role of the known posttranslational modifications of this protein sulfation (49) and phosphorylation (50), (51) (shown in Fig. S1) remains to be established.

In summary, our data provide new insight into the function of neuronal 7B2 and establish this protein as a novel anti-aggregation chaperone strongly associated with neurodegenerative disease. Interestingly, recent proteomics studies also point to an association of 7B2 with various neurodegeneration-related protein misfolding diseases, including AD, PD and ALS (20); (21); (22); (23). These observations are supported by data showing that 7B2 is significantly upregulated in brain tissues of AD patients (52) and by a fifth proteomics study in which 7B2 was found to be elevated in the cerebrospinal fluid of ALS patients (53) indicating possible up-regulation of 7B2 as a response to increased protein aggregation and misfolding. Taken together with the work presented here, these studies provide support for the idea that 7B2
plays a role in the etiology of aggregate formation in neurodegenerative disease.

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FIGURE LEGENDS

Figure 1: 7B2 colocalizes with amyloid plaque pathology. (A) The hippocampus of a 12-month-old mutant APP-PSEN1 mouse strongly stains for Aβ1-42-immunoreactivity. A composite image shows significant overlap between Aβ1-42 (green) and 7B2 (red) immunoreactivity resulting in yellow color in the merged image (arrows). Scale bar is 100 μm. (B) Staining showing significant colocalization (overlap resulting in purple hue) of 7B2 (red) with Aβ1-42 (green) and genuine extracellular plaques (blue) in the cortex of APP-PSEN1 mice. Plaques are visualized by staining with the in vivo amyloid-imaging fluorophore methoxy-X04.

Figure 2: Co-localization of 7B2 with extra- and intracellular protein aggregates in the Alzheimer’s hippocampus and in Lewy bodies of Parkinson’s disease. (A) Alzheimer’s brain: Schematic representation of 7B2-immunoreactivity (red stars) and Aβ-positive plaques (green stars), as detected throughout the extent of the human brain sample at the level of the hippocampus. Low magnification images provide an overview of the areas of Aβ-immunoreactive deposits (red) and 7B2 (green) expression within in the hippocampus. High magnification images of representative amyloid plaques within the hippocampus confirm a high degree of colocalization (arrow) of 7B2-immunoreactivity with Aβ-immunoreactivity. (B) Parkinson’s brain: 7B2-immunoreactivity (red stars) were found throughout the mesencephalon while Lewy bodies were confined to the substantia nigra (green stars). Upper panel: Low magnification images provide an overview of the areas of α-synuclein-immunoreactive deposits in Lewy bodies (green) and 7B2 expression (red) within the substantia nigra. Lower panel: High magnification images of representative Lewy bodies within the substantia nigra confirm a high degree of colocalization (arrow) of 7B2-immunoreactivity with α-synuclein-immunoreactivity. The majority of 7B2-immunoreactivity was confined to areas near the nucleus, suggesting intracellular localization. (C) 7B2-immunoreactivity was detected in a human control brain sample; shown are representative images of the hippocampus in a non-diseased control brain. While only limited Aβ-immunoreactivity (red) and no plaque burden was detected, we observed significant 7B2-immunoreactivity (green) that was confined to areas near cell nuclei, suggesting intracellular localization. CA, cerebral aqueduct; CT, corticopontine tract; Hp, hippocampus; LN, lentiform nucleus; MRF, mesencephalic reticular formation; PT, pyramidal tract; RN, red nucleus; SN, substantia nigra; TH; thalamus. Scale bar, 10 μm.

Figure 3: 7B2 decreases Aβ-induced cell death in Neuro-2A cells. (A) Neuro-2A cells were treated with 10 μM Aβ1-42 for 48 h to induce cell death in the presence or absence of 7B2. Left panel:
Quantification of Aβ-induced cell death by WST-1 cell viability assay. **Right panel:** Representative photomicrographs showing viable calcein AM-stained Neuro-2A cells following treatment with Aβ1-42 with or without 7B2. (B) Quantification of endogenous 7B2 levels in Neuro-2A cells by RIA following either adenoviral overexpression or RNAi-mediated knock-down. (C) Aβ1-42-induced cell death following manipulation of intracellular 7B2 levels was monitored using the WST-1 cell viability assay. (D) Exogenously added recombinant His-tagged 7B2 (green arrows) is internalized and colocalizes with fluor-labeled Aβ1-42 (red) in Neuro-2A cells, indicating co-uptake into the cytosol of Neuro-2A cells (yellow arrows). His-tag antiserum was used for this experiment.

**Figure 4:** Structure-function analysis of 7B2 proteins in suppressing Aβ fibrillation. (A) Domain structure of 7B2 and schematic representation of the amino-terminal deletions and peptides used in this study. (B) Aβ1-42 (20 μM) was incubated with either full-length 7B2 (27 kDa, red) or the smaller truncated proteins and peptides (2 μM). Protein fibrillation was monitored using a ThT fibrillation assay. (C) The inhibition of Aβ1-42 aggregation in the presence of 27 kDa 7B2 was dose-dependent and was most effective at a molar ratio of 1:10 (7B2 : Aβ1-42). (D) Quantification of supernatant (soluble Aβ1-42) vs. pellet (insoluble Aβ1-42) dot intensities revealed a ratio shift (supernatant : pellet) towards soluble Aβ1-42 species following addition of 7B2. (E) Quantification of Aβ1-42 fibril formation observed after 72 h incubation in reactions with or without 7B2 by transmission electron microscopy.

**Figure 5:** 7B2 does not disintegrate preformed mature Aβ1-42 fibrils but suppresses Aβ1-40 and α-synuclein fibrillation. (A) Aβ1-42 (20 μM) was incubated at 37 °C followed by addition of 2 μM 27 or 21 kDa 7B2 at a time point indicated by arrow. Protein aggregation was monitored by ThT fibrillation assay. Further Aβ1-42 aggregation was inhibited once 7B2 was added, however, pre-formed mature fibrils were not affected (n = 3/group). Amyloid beta1-40 (20 μM) (B) and α-synuclein (44 μM) (C) were incubated with full-length 7B2 (27 kDa, red) or 21 kDa 7B2 (blue) respectively and fibrillation monitored by the ThT assay. (D) Dose-dependence relationship for inhibition of α-synuclein fibrillation by 27 kDa 7B2.

**Figure S1:** 7B2 structure, lack of chaperone-like refolding activity and lack of molecular similarity to chaperones in the small heat shock protein (sHSPs) family. (A) Amino acid sequence of rat 7B2 including N-terminal signal peptide and the C-terminal inhibitory peptide domain. Putative posttranslational modification sites are marked (red P, known phosphorylation site, blue P, hypothetical phosphorylation site, S, sulfation; S-S, disulfide bond) and the C-terminal cleavage site is underlined; the minimal amino acid sequence required for PC2 activity is boxed. The first and last three amino acids of
the 7B2 fragments used in this study are indicated in bold letters. Green asterisks indicate the beginning and the end of the amino acid sequence used for sequence alignment with α-crystallin. (B) Unfolded and inactive firefly luciferase (40 μM) was incubated with 7B2 (4 μM), followed by measurement of regained (refolded) luciferase enzyme activity, as determined by luciferin bioluminescence assay. (C) Best multi-sequence alignment of human α-crystallin with heat shock protein 27 (HSP27) and 7B2. The heat shock protein-characteristic α-crystallin domain (ACD) is indicated in green.
Reference List


Alzheimer's hippocampus

Aβ1-42 merge

DAPI

merge

7B2

merge

C
non-disease control (Hippocampus)

Parkinson's substantia nigra

merge

α-Syn

merge

Aβ1-42

merge

Fig. 2 Helwig et al.

A

Calcine AM cell viability staining

Control
Aβ_1-42

+ 27 kDa 7B2

B

C

D

Helwig et al.

Fig. 3
Fig. 4

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Michael Helwig, Akina Hoshino, Casey Berridge, Sang-Nam Lee, Nicolai Lorenzen, Daniel E. Otzen, Jason L. Eriksen and Iris Lindberg

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