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Original Publication:
http://dx.doi.org/10.1016/j.nbd.2015.08.024
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Postprint available at: Linköping University Electronic Press
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-122341
Protective properties of lysozyme on β-amyloid pathology: implications for Alzheimer disease

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Abstract

The hallmarks of Alzheimer disease are amyloid-β plaques and neurofibrillary tangles accompanied by signs of neuroinflammation. Lysozyme is a major player in the innate immune system and has recently been shown to prevent the aggregation of amyloid-β1-40 in vitro. In this study we found that patients with Alzheimer disease have increased lysozyme levels in the cerebrospinal fluid and lysozyme co-localized with amyloid-β in plaques. In Drosophila neuronal co-expression of lysozyme and amyloid-β1-42 reduced the formation of soluble and insoluble amyloid-β species, prolonged survival and improved the activity of amyloid-β1-42 transgenic flies. This suggests that lysozyme levels rise in Alzheimer disease as a compensatory response to amyloid-β increases and aggregation. In support of this, in vitro aggregation assays revealed that lysozyme associates with amyloid-β1-42 and alters its aggregation pathway to counteract the formation of toxic amyloid-β species. Overall, these studies establish a protective role for lysozyme against amyloid-β associated toxicities and identity increased lysozyme in patients with Alzheimer disease. Therefore, lysozyme has potential as a new biomarker as well as a therapeutic target for Alzheimer disease.

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1. Introduction

Alzheimer disease is the primary cause of dementia and is manifested as an accumulation of oligomers and extracellular plaques composed of amyloid-β (Aβ), intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau, synaptic failure and progressive neurodegeneration (Blennow et al., 2006). There are several risk factors proposed for sporadic Alzheimer disease, with ageing and neuroinflammation suggested to be important contributors to the disease (Akiyama et al., 2000; Heneka and O'Banion, 2007; McGeer and McGeer, 2007). There is evidence that Aβ deposits in the brain trigger neuroinflammation via activation of the microglia and astrocytes that surround amyloid plaques (Halle et al., 2008; Wyss-Coray and Rogers, 2012). However, whether inflammation is the driving force, a contributor or a secondary effect of Alzheimer disease pathology is not known. Sequential cleavage of the amyloid precursor protein (APP) generates Aβ1-42, and the accumulation of Aβ is central to Alzheimer disease pathogenesis (Hardy and Selkoe, 2002). Several Aβ isoforms are observed, with Aβ1-40, Aβ1-42, Aβ1-43 and the N-terminally truncated isoforms Aβ3-42 and Aβ11-42 being neurotoxic (Jonson et al., 2015) and Aβ1-42 is the most abundant isoform during Alzheimer disease and is highly aggregation prone. During Aβ aggregation, monomers turn into oligomers, prefibrillar species, fibrils and insoluble plaques. Aβ aggregation is characterized by a lag phase in which seeds are formed and a logarithmic phase in which the seeds elongate into fibrils that catalyze further fibrillation, which in turn accelerates the aggregation process (Wogulis et al., 2005). In the Alzheimer disease brain, Aβ1-42 is the main constituent of plaques (Portelius et al., 2010). Intermediate structures of aggregated Aβ species generated prior to the formation of the insoluble plaques; oligomers are cytotoxic and cause phosphorylation of tau, synaptic failure and neuronal death (Lesne et al., 2013). Several endogenous proteins can manipulate the aggregation process of Aβ. The physical interaction between...
Aβ and the chaperones clusterin, αB-crystalline, haptoglobin, and α2-macroglobulin can inhibit Aβ aggregation; thus, these chaperones may play an important role in Alzheimer disease pathogenesis (Matsubara et al., 1996; Du et al., 1998; Barral et al., 2004; Raman et al., 2005; Yerbury et al., 2009).

Lysozyme is part of the innate immune system and possesses bacteriolytic properties capable of hydrolyzing peptidoglycans in the bacteria cell wall (Fleming, 1922). Lysozyme is secreted by epithelial cells, macrophages, astrocytes and microglia, and the enzyme is abundant in various tissues and fluids including liver, spleen, milk, tears, saliva and CSF (Ganz, 2004). Lysozyme retains both anti-oxidant and anti-inflammatory properties (Ogundele, 1998; Liu et al., 2006; Lee et al., 2009), and the level of lysozyme has been reported to be increased in CSF during inflammation (Hällgren and Venge, 1982). Lysozyme was recently shown to inhibit Aβ1-40 aggregation via binding to the monomeric form of Aβ1-40 (Luo et al., 2013; Luo et al., 2014) and has also been predicted to prevent the aggregation of Aβ1-42 (Luo et al., 2013; Luo et al., 2014) and has also been predicted to prevent the aggregation of Aβ1-40 (Luo et al., 2013; Luo et al., 2014). This may be neuroprotective for increasing Aβ toxicity identifying a potential new therapeutic pathway for the disease.

2. Materials and methods

2.1. Human study populations

2.1.1. CSF cohorts and lysozyme measurements

This study was performed on two separate sets of de-identified, archived matched CSF and serum samples. The study was approved by the Ethical committee at the University of Gothenburg. All samples were provided by the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital/Möln达尔, Sweden. The samples were collected from patients who sought medical advice because of minor cognitive or neurological symptoms. Following routine clinical assessment, the CSF biomarkers for Alzheimer’s disease; P-tau181P, T-tau and Aβ1-42 (Hansson et al., 2006) were analyzed in these patients, and based on their neurochemical profile, the samples were divided into three groups; those with CSF biomarkers in the Alzheimer’s disease range (AD, n = 10), those with CSF biomarkers in the control range (C, n = 10) and those with CSF biomarkers indicative of neuronal injury but not Alzheimer’s disease (having high T-tau, n = 5). The samples with Alzheimer’s disease were designated according to CSF biomarker levels using cutoffs that have 95% sensitivity and 80% specificity, including T-tau 350 ng/l, Aβ1-42 530 ng/l and P-tau181P 60 ng/l (Hansson et al., 2006). For further description of patient information, CSF collection and protein analysis via ELISA and Western blotting, see the previously described methods (Armstrong et al., 2014). The primary antibodies used for Western blotting were lysozyme (A0099, Dako, Glostrup, Denmark) and GAPDH (247 002, Synaptic Systems GmbH, Göttingen, Germany). Equal sample loading was verified by Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining of total protein in each lane on the membranes. The films were scanned and the immunoblots were quantified using the Image J program (available at http://rsbweb.nih.gov/ij/). The relative amount of protein corresponding to an immunoreactive band was calculated as a product of average optical density of the area of the band.

2.1.2. Autopsy confirmed cohorts with brain samples and tissue localization methods

Tissues were received from the Sydney Brain Bank at Neuroscience Research Australia and the New South Wales Tissue Resource Centre at the University of Sydney which are supported by the National Health and Medical Research Council of Australia (NHMRC), University of New South Wales, Neuroscience Research Australia, Schizophrenia Research Institute and National Institute of Alcohol Abuse and Alcoholism (NIH (NIAAA) R24AA012725). Brain tissue collection procedures were approved by the University of New South Wales Human Research Ethics Committee and informed consent was obtained from all individuals prior to donation of their brain. Cases were selected based on their characterization with Alzheimer disease according to established neuropathological criteria (Montine et al., 2012).

For immunohistochemistry, formalin-fixed, paraffin-embedded 10 μm sections of inferior temporal cortex tissues underwent heat-induced antigen retrieval with citrate buffer (pH 6.0) for 3 min, followed by formic acid pretreatment for 3 min. Then, sections were sequentially double-labeled with rabbit monoclonal anti-human lysozyme (1:100, Abcam, Cambridge, UK) visualized with the NovoLink Polymer Detection System (RE7150-K, Leica Biosystems, Wetzlar, Germany). Double labeling was performed with the lysosome antibody and mouse monoclonal anti-human Aβ1-42 (1:200, M0872, Dako, Glostrup, Denmark) visualized with donkey anti-rabbit Alexa Fluor 568 (1:250, A-10,042, Molecular Probes, Waltham, MA, USA) and goat anti-mouse Alexa Fluor® 488 (1:500, A-11,001, Molecular Probes, Waltham, MA, USA) respectively. Sections were coverslipped with VectaShield for fluorescence (H-1000, Vector, Burlingame, CA, USA), and examined on a confocal fluorescence microscope (Nikon C1si). Control experiments for the double labeling were performed with only a single or no primary antibody with the result of single or no protein labeling observed.

2.1.3. Lysozyme detection by Mesoscale Discovery (MSD)

The MSD technique is similar to the ELISA technique but with electrochemiluminescent detection of the analytes. A standard binding 96-well multi-array plate (L15XA-6, MSD) was coated with 25 μl of 15 μg/ml cAbHuL6 (the N-terminal domain of a camelid heavy-chain antibody specific for human lysozyme (Dumoulin et al., 2003) (1 h, RT), then the plate was washed with 150 μl PBS-T. A total of 25 μl block solution (1% milk in PBS-T) was added to the wells and incubated (1 h, RT), then 20 μl CSF was added to the plate and incubated (1 h, RT). The plate was washed and 25 μl rabbit anti-human lysozyme antiserum (1:1000 in 1% milk in PBS-T) was added to the wells and incubated (1 h, RT). The plate was washed, and 25 μl Sulfo-Tag goat anti-rabbit antibody (R32AB-5, MSD, 1:500 in 1% milk in PBS-T) was added and the plate was incubated (1 h, RT). The plate was washed, and 150 μl 2X read buffer (R92TC-2, MSD) was added. The plate was analyzed using a SECTOR imager 2400 instrument (MSD).

Ten fly heads of each genotype were snap-frozen, 150 μl PBS-PI (PBS buffer with Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Basel, Switzerland) was added and the fly heads were homogenized. The samples were centrifuged (13,000 rpm, 1 min); the supernatant constituted the soluble fraction. Lysozyme levels were detected as described above.

2.1.4. Cell culture

Human SH-SY5Y neuroblastoma cells were cultured in Dulbecco’s Modified Eagle Medium:Nutrient Mixture F-12 GlutaMAX™ supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% MEM non-essential amino acids solution and 10% fetal bovine serum in a humidified atmosphere with 5% CO2. To achieve a neuronal-like phenotype, the cells were pre-treated with 10 μM retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) for 7 days prior to the experiments.
viability was determined by the XTT assay (Roche) according to the manufacturer's instructions. The XTT absorbance was measured at 450–750 nm using a Victor Wallac plate reader (PerkinElmer, Waltham, MA, USA). Human neuroglioma H2APPswc cells were cultured in Opti-MEM® supplemented with 100 μ/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml blasticidin, 200 μg/ml hygromycin B and 10% fetal bovine serum in a humidified atmosphere with 5% CO2.

2.1.5. Oligomeric Aβ1-42 preparation

Freshly made oligomeric Aβ1-42 was prepared for each experiment. Recombinant Aβ1-42 (rPeptide) was lyophilized in hexafluoropropanol (HFIP) and dissolved in DMSO to 1.5 mM. Aβ1-42 was vortexed and diluted with cold HEPES buffer to a concentration of 100 μM, vortexed 30 s, then sonicated 2 min and incubated 24 h at 4°C. Size exclusion chromatography and electron microscopy confirmed the formation of oligomers (Domett et al., 2014).

2.1.6. Fluorescent labeling of lysozyme

The free amine of the lysine on lysozyme was labeled with the Alexa Fluor® 488 protein labeling kit (Life Technologies). Sodium bicarbonate buffer (pH 8.5) was used to prevent aggregation during the labeling process. The labeled proteins were separated from the free dye by gel chromatography using a PD-10 column eluted with HEPES buffer at pH 7.4. The concentration of Alexa-488-labeled lysozyme was measured on a Nanodrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden) and calculated by subtracting the contribution of Alexa-488 at 280 nm from the observed absorbance using the following equation:

\[
[A280(lysoyme)] = \frac{A_{280}(observed) - A_{495}(Alexa 488)}{495(Alexa 488)}
\]

The concentration was calculated using the extinction coefficient of lysozyme as 2.64 ml mg⁻¹ cm⁻¹ at 280 nm and the 71,000 cm⁻¹ M⁻¹ M extinction coefficient of the Alexa 488 fluor probe. The calculated labeling percentage was ~58%.

2.1.7. Fluorescence resonance energy transfer (FRET) using steady state fluorescence spectroscopy

The intermolecular interaction between lysozyme and Aβ1 species was studied by FRET using steady-state fluorescence spectroscopy. Fluorescence emission spectra measurements were performed in a Tecan Safire2 multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). Alexa-488 (Exmax 494 nm and Emmax 520 nm)-labeled lysozyme was used as the donor and N-terminal TAMRA (carboxytetramethylrhodamine)-labeled synthetic Aβ1-42 (Innovagen, Lund, Sweden) as the acceptor (Exmax 555 nm and Emmax 580 nm) (Biskup et al., 2007, Koch et al., 2008). Before the measurements, lysozyme-Alexa-488 (200 nM) was incubated for 1 h at 37 °C with 8 μM of monomeric Aβ1-TAMRA in HEPES buffer (pH 7.4). Size exclusion chromatography confirmed that more than 99% of the Aβ1-TAMRA was monomers using a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in PBS.

2.1.8. ThT aggregation assay

Recombinant Aβ1-42 (rPeptide) lyophilized from HFIP was dissolved in 2 mM NaOH to a concentration of 222 μM. Aβ1-42 was diluted to a final concentration of 10 μM and then aggregated alone or with 10 or 40 μM lysozyme (Sigma-Aldrich) in the presence of 0.3 μM ThT. The samples were loaded in a Corning 96-well black-well clear-bottom microtiter plate (Corning Inc. Life Sciences, Tewksbury, MA, USA), and the in situ change in ThT fluorescence was recorded under quiescent conditions at 37 °C for 24 h using fluorescence spectroscopy (Tecan). The excitation was performed at 440 nm and the emission intensity were recorded at 480 nm. For the ThT curves of Aβ1 aggregated alone or in the presence of lysozyme (10 μM), the rate of aggregation (k) was extracted from the maximal slope of the sigmoidal curve and the length of the lag time (t1) was determined by fitting the initial data to a straight line and a tangent to the steepest region of the growth curve; t1 is defined as the time point where the straight line and the tangent intersect.

2.1.9. Transmission electron microscopy (TEM) of end products from the ThT aggregation assay

Samples collected from the 24 h ThT aggregation assay were snap-frozen in liquid nitrogen. A total of 10 μl of each sample was added to formvar/carbon-coated 400 mesh copper transmission electron microscopy grids (Agar Scientific Ltd., Stansted, UK) for 2 min. The fluid was removed and 10 μl of 4% uranyl acetate was added for 2 min. The grids were analyzed with a JEOL JEM1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan) equipped with a SC1000 ORIUS CCD camera and the DigitalMicrograph (DM) v.1.7.1.38 software (Gatan Inc., Pleasanton, CA, USA).

2.1.10. Immunoanalyses of end products from the ThT aggregation assay

A total of 20 μl of the 24 h product formed in the ThT aggregation assay was transferred for each reaction. The samples were incubated for 1 h at RT with an antibody solution containing mouse monoclonal anti-Aβ antibody (1:500 in PBS; Mabtech, Nacka strand, Sweden) and rabbit monoclonal anti-lysozyme antibody (1:500 in PBS; Abcam). The samples were centrifuged for 10 min at 13,000 rpm and the supernatant was discarded. The pellet was resuspended in a secondary antibody solution containing fluorescently labeled goat anti-mouse (1:600 in PBS; Alexa Fluor 594 goat anti-mouse; Life technologies) and fluorescently labeled goat anti-rabbit (1:600 in PBS; Alexa Fluor 488 goat anti-rabbit; Life technologies) and incubated for 1 h at RT. The samples were centrifuged for 10 min at 13,000 rpm and the supernatant was discarded. A total of 5 μl of the pellet was transferred onto SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and allowed to dry. The slides were mounted using fluorescence mounting medium (Dako) and analyzed using a Zeiss LSM 780 confocal microscope (Zeiss, Oberkochen, Germany). The ELISA analyses of the 24 h end point samples were performed by a previously described method that specifically detects the aggregated forms of Aβ1 without detecting monomers (Holitta et al., 2013). The same monoclonal anti-Aβ1 N-terminal specific 82E1 antibody (JBL International, Hamburg, Germany) was used both for capture and detection.

2.1.11. Fly stocks

The Gal4/UAS system was used for CNS-specific expression of UAS transgenes in Drosophila melanogaster (Brand and Perrimon, 1993). The C155-gal4 driver (Lin and Goodman, 1994) was used to express the transgenes in the CNS. Strains of C155-gal4 flies carrying UAS-containing genes encoding human wild type lysozyme (Kumita et al., 2012), Aβ1-42 (Crowther et al., 2005) and both lysozyme and Aβ1-42 (both containing a secretion-tag) were generated. Background C155-gal4 w1118 (178) was used to generate control flies. D. melanogaster stocks were allowed to develop under a 12:12 h light:dark cycle at 29 °C at 60% humidity. The flies were kept in 50 ml plastic vials containing standard Drosophila food (corn meal, molasses, yeast and agar) and maintained post-eclosion in 50 ml plastic vials containing six ml agar (20 g agar and 20 g sugar dissolved in 11 water) and yeast paste (dry Baker's yeast dissolved in water) at 29 °C under 12:12 h light:dark cycle.

2.1.12. Drosophila longevity and locomotor assays

One hundred Drosophila flies of each genotype were divided into plastic vials containing agar food and yeast paste with ten flies in each vial and kept at 29 °C in a 12:12 h light:dark cycle. Every 2–3 days the flies were transferred to fresh food and the numbers of flies alive were counted. Prism GraphPad software 6 (GraphPad Software, San Diego, CA USA) was utilized to graph Kaplan–Meier survival curves (Kaplan and Meier, 1958) and to run the log rank statistical analysis.

To analyze the locomotor behavior of the flies, a locomotor assay using the iFly software (Jahn et al., 2011), was used. For each genotype, three vials with ten flies in each were collected and filmed for 90 s. The
flies were tapped to the bottom every 30 s (re-activating the locomotor behavior), yielding three video clips with three clips of 30 s each. The videos were processed and analyzed using the iFly software, which calculated the velocities and the angle of movement for each clip. The data were plotted using GraphPad Prism 6.

### 2.1.13. Immunohistochemistry of fly heads

**Drosophila** heads were embedded on the day of eclosion or at day 20 in Tissue-Tek OCT compound (Histolab, Gothenburg, Sweden) using Cryomold specimen molds and stored at —80 °C until use. The OCT blocks were sectioned using a Microm HM 550 Cryostat (Microm International, Walldorf, Germany) into 20 μm thin sections that were placed on SuperFrost Plus slides (Menzel-Gläser) and stored at —20 °C until use. The sections were fixed in 4% w/v PFA in PBS for 10 min at RT. The slides were washed in PBS (3 × 3 min) followed by a (1 × 3 min) wash in PBS-T (PBS with 0.05% w/v Tween-20) before blocking in 10% w/v BSA in PBS-T for 1 h at RT. Mouse monoclonal anti-Aβ antibody (1:500 in 1% BSA w/v in PBS-T; Mabtech) and rabbit monoclonal anti-lysozyme (1:500 in 1% BSA w/v in PBS-T; Abcam) were added to the slides and incubated for 1 h at RT. The slides were washed with PBS-T (3 × 5 min). Goat anti-mouse fluorescently labeled secondary antibody (1:600 in 1% w/v BSA in PBS-T; Alexa Fluor 488 goat anti-mouse; Life Technologies) and goat anti-rabbit fluorescently labeled secondary antibody (1:600 in 1% w/v BSA in PBS-T; Alexa Fluor 488 goat anti-rabbit; Life Technologies) were applied to the slides and incubated for 1 h at RT. The slides were mounted using Vectorshield with DAPI (Vector Laboratories). The slides were analyzed using a Zeiss LSM 780 confocal microscope (Zeiss). The micrographs were processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA); all images were treated identically.

### 2.1.14. Analyses of Aβ1-42 levels in Drosophila

Five fly heads for each genotype were placed in Eppendorf tubes and snap frozen, and then analyzed on MSD as described previously (Caesar et al., 2012). To adjust for variation in the protein extraction step, a quantitation of the total amount of protein from each sample of fly homogenate was performed using the Bio-Rad DC Protein Assay Kit II (500–0112, Bio-Rad, Hercules, CA, USA).

### 2.1.15. Lysozyme expression in cells and binding to fibrillar Aβ

Neuroblastoma and neuroglioma cells were incubated with oligomeric Aβ1-42 diluted in serum-free culture media at a final concentration of 1 μM for 1 h. After extensive PBS washing and trypsinization, the cells were re-seeded and grown in serum-free media for 24 or 48 h. The cells were lysed, and lysozyme levels were measured via Western blotting as described previously (Armstrong et al., 2014).

A total of 50 μg recombinant Aβ1-42-TAMRA lyophilized from HFIP was dissolved in 2.2 μL DMSO, diluted to 100 μM in 20 mM HEPES (pH 7.4), vortexed, and aggregated at 37 °C for 24 h. The aggregated fibrillar Aβ1-42-TAMRA was diluted to a final concentration of 10 μM and incubated 1 h at RT alone or with 40 μM lysozyme-Alexa 488 (Sigma-Aldrich). The samples were centrifuged for 10 min at 13,000 rpm and the supernatant was discarded. A total of 5 μL of the pellet was transferred onto SuperFrost Plus slides (Menzel-Gläser) and allowed to dry. The slides were mounted using fluorescence mounting medium (Dako) and sealed with nail Polish and were analyzed using a Zeiss LSM 510 META confocal microscope (Zeiss).

### 2.2. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software.

The mean value and standard deviation or standard error of the mean were calculated for all data. The nonparametric Mann–Whitney U test was used to test for significant differences between groups. For Drosophila longevity statistics, Kaplan–Meier survival curves and log-rank (Mantel–Cox) statistical analyses were performed. Statistical significance was defined for p-values less than or equal to 0.05 (*), 0.01 (**) and 0.001 (***)

### 3. Results

#### 3.1. Lysozyme is increased in CSF and present in plaques from patients with Alzheimer disease

Lysozyme levels were tested in two cohorts of CSF samples collected from patients with Alzheimer disease and controls (Table 1, CSF set 1 and 2). Lysozyme levels were significantly higher in the CSF samples from Alzheimer patients (Fig. 1A and B). This was demonstrated using two different methods: Western blotting with quantification as shown in Fig. 1A and the Meso Scale Discovery technique for the second validation cohort of CSF samples as shown in Fig. 1B. Lysozyme levels did not differ between the control and Alzheimer disease cohorts in matched serum samples (Fig. 1B). To investigate whether lysozyme up-regulation was due to general neurodegeneration, lysozyme levels were analyzed in the CSF of a third set of non-Alzheimer disease patients who had normal Aβ1-42 and P-tau181P levels but elevated total tau (T-tau). No significant difference between lysozyme levels were

### Table 1

<table>
<thead>
<tr>
<th>Study group</th>
<th>Age years</th>
<th>Sex M:F</th>
<th>CSF T-tau ng/l</th>
<th>CSF Aβ42 ng/l</th>
<th>CSF P-tau181P ng/l</th>
<th>CSF/serum Albumin ratio</th>
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</thead>
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<tr>
<td><strong>CSF set 1</strong></td>
<td></td>
<td></td>
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<tr>
<td>AD n = 10</td>
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<td>5:5</td>
<td>880</td>
<td>330</td>
<td>123</td>
<td>5.5</td>
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<tr>
<td>C n = 10</td>
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<td>6:4</td>
<td>295</td>
<td>780</td>
<td>42</td>
<td>7.8</td>
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<tr>
<td>AD n = 10</td>
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<td>844</td>
<td>342</td>
<td>97</td>
<td>7.6</td>
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<tr>
<td>C n = 10</td>
<td>64</td>
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<td>424</td>
<td>821</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C 1-5 set 1</td>
<td>72</td>
<td>3:2</td>
<td>271</td>
<td>705</td>
<td>42</td>
<td>6.7</td>
</tr>
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<td>n = 5</td>
<td>65.2</td>
<td>2:3</td>
<td>2541</td>
<td>466</td>
<td>73</td>
<td>No data available</td>
</tr>
</tbody>
</table>
detected between the control and high-T-tau samples (Fig. 1C), demonstrating that the increase in lysozyme in Alzheimer disease CSF was not caused by generalized neuronal damage.

To study whether lysozyme and Aβ1-42 co-localize in plaques in Alzheimer disease, immunohistochemistry was performed using postmortem formalin-fixed and paraffin-embedded inferior temporal
cortex tissues from patients with sporadic Alzheimer disease. The staining pattern of lysozyme resembled the morphology of diffuse and neuritic plaques (Fig. 2A and B). Double-labeling with antibodies against lysozyme and Aβ1–42 revealed that lysozyme was localized with Aβ1–42 in diffuse and neuritic plaques, with clumping of lysozyme around the plaque core (Fig. 2C–H).

In the CSF from patients with Alzheimer’s disease there was no correlation between the levels of Aβ1–42 and lysozyme, largely due to the similar concentrations of Aβ1–42 (310–390 ng/l) in the CSF samples at this stage of the Alzheimer’s disease. In order to explore whether Aβ could up-regulate the expression and secretion of lysozyme in cell models, both neuroglioma cells and differentiated human neuroblastoma cells were exposed for 24 h or 24 and 48 h, respectively, to a subtoxic concentration of 1 μM oligomeric Aβ1–42. A significant increase in both intracellular lysozyme and secreted lysozyme was detected from the neuroglioma cells (Fig. 3A–B). In the neuroblastoma cells, secreted lysozyme was not detected in the medium (data not shown) and the intracellular level of lysozyme was not changed after 24 h, but was significantly increased after 48 h of Aβ1–42 exposure (Fig. 3C). This result demonstrates that Aβ can trigger the expression of lysozyme in cell-based systems and indicates that the increased levels of lysozyme in CSF from Alzheimer disease patients might originate from gliacells.

3.2. Lysozyme suppresses the toxicity of Aβ in D. melanogaster

On the basis of these clinical results, we developed a novel lysozyme/Aβ1–42 Drosophila line by co-expressing human lysozyme with Aβ1–42 (lysozyme:Aβ) in the CNS using the neuronal-specific C155-gal4 driver. Both lysozyme and Aβ1–42 were expressed with a secretion tag to allow secretion into the extracellular space. A longevity assay was performed to monitor the toxic effects of the Aβ peptide on fly neurons (Crowther et al., 2005; Hirth, 2010) and to investigate the impact of lysozyme on in vivo Aβ toxicity. The median survival time (represented by the

**Fig. 3.** Lysozyme is up-regulated in neuroglioma and neuroblastoma cells treated with oligomeric Aβ. Intracellular and secreted levels of lysozyme in cells treated with 1 μM oligomeric Aβ for 24 or 48 h were analyzed using Western blot. Densitometric quantifications were performed on the scanned Western blots and normalized to GAPDH levels or Ponceau S staining. The bars represent the mean ± SD, n = 3 (*p < 0.05). Blots were cropped. A) Lysozyme levels in neuroglioma H4APPswe cells, B) H4APPswe supernatants and C) differentiated SH-SYSY neuroblastoma cells.
death of 50% of the flies) was 28 days for the Aβ flies and 32 days for both the control and lysozyme:Aβ flies (Fig. 4A). Thus, co-expressing lysozyme with Aβ resulted in an increased life span of the Aβ flies that equaled the median survival time of the control flies. To achieve a more complete picture of fly health, a locomotor assay was performed using the iFly technique (Jahn et al., 2011) to analyze the velocity and angle of movement. The average velocity for a healthy young fly is 10 mm/s; as the fly ages, the velocity decreases. Shortly before the flies die they become immobile and their velocity cannot be recorded; thus, a cut-off value of 4 mm/s was set as an indication of disability. The Aβ-expressing flies showed a substantial reduction in their velocity, reaching the cut-off value of 4 mm/s at day 20 compared with day 35 for the control flies (Fig. 4B), when co-expressing lysozyme with Aβ, this effect was postponed by 6 days and occurred at day 26 (Fig. 4B). The angle of movement describes the deviation of the flies’ path from vertical when they move from the bottom to the top of the vial (Khabirova, 2012). The angle of movement for a healthy young fly is approximately 55°, and this angle increases as the fly ages. A cut-off value of 80° was set where values above indicate that the mobility functions of the flies (i.e., orientation and movement direction) are impaired. For the Aβ flies, the angle of movement diverted from that of healthy control flies at day 14 when the cut-off value of 80° was reached. However, for the lysozyme:Aβ flies this day was postponed by 7 days, and the cut-off value was not reached until day 21. The control flies demonstrated a normal angle of movement until day 34 (Fig. 4C). The protective effects of lysozyme in the longevity and locomotor assays of the Aβ1-42 expressing flies revealed that lysozyme has a pathomechanistic relevant effect on the reduction of Aβ toxicity.

To investigate the location of Aβ and lysozyme in the fly CNS, sections of Drosophila brains expressing lysozyme, Aβ or both were stained with antibodies against lysozyme and Aβ1-42. In flies co-expressing lysozyme and Aβ1-42 the proteins were in close proximity. This finding strongly suggests an interaction between these two proteins in the fly CNS (Fig. 5).

The effects of lysozyme on the levels of Aβ1-42 in Drosophila brains were also analyzed. Soluble and insoluble Aβ1-42 levels were measured in the Aβ flies with and without co-expression of lysozyme at the day of eclosion (day 0) and 20 days later. For Aβ1-42 expressing flies, the insoluble fraction of Aβ1-42 was higher than the soluble fraction both at days 0 and 20 (Fig. 6A, B); indeed these insoluble species accumulated over time, and a significantly higher level was detected at day 20 compared with day 0 (Fig. 6B). When co-expressing lysozyme and Aβ1-42, the accumulation of both soluble and insoluble Aβ1-42 in aged flies was significantly reduced compared with flies expressing Aβ1-42 alone (Fig. 6A, B). A reduced level of lysozyme was detected both at day 0 and day 20 in the Aβ flies; this difference was highly significant at day 20 (Fig. 6C). These analyses clearly show that lysozyme and Aβ are able to interact in the fly CNS and that the presence of lysozyme results in decreased amounts of accumulated Aβ species in the Aβ1-42 flies.

### 3.3. Lysozyme binds to and inhibits the Aβ fibril process and cell death

Next, we studied the in vitro interaction properties between lysozyme and Aβ using FRET steady state fluorescence measurements. The decrease of donor fluorescence at 520 nm from lysozyme labeled with Alexa-488 and the increase of acceptor fluorescence at 580 nm from monomeric Aβ1-42 labeled with TAMRA demonstrated FRET between the donor and acceptor due to binding of lysozyme with monomeric Aβ1-42 (Fig. 7A). To investigate the capacity of lysozyme to interact with fibrillar Aβ, 10 μM of TAMRA-labeled Aβ1-42 was aggregated for 24 h prior to the addition of Alexa-488—labeled lysozyme (40 μM) for 1 h. Aβ was detected as fibrillar structures, and lysozyme was found associated with these structures (Fig. 7B).

To investigate whether the binding between lysozyme and Aβ has any effect on the aggregation kinetics of Aβ, the fluorescence signal from thioflavin T (ThT) (Borrs et al., 2010) was followed during the aggregation of 10 μM Aβ1-42 in the absence and presence of lysozyme. The Aβ aggregation kinetics were substantially slowed down at a 1:1 ratio between Aβ1-42 and lysozyme; the rate of aggregation (k), which is a measure of the elongation efficiency of fibril formation, was decreased from 17 min⁻¹ to 5 min⁻¹ and the lag time (tₜ), which mirrors the rate of nucleation events, was prolonged from 4 h to 6 h (Fig. 8A). The end ThT signal was also greatly reduced from 7800 AU for Aβ alone aggregated, to 4200 AU in the presence of an equal concentration of lysozyme and to 450 AU in the presence of a four-fold higher concentration of lysozyme (Fig. 8A). No change in the ThT signal was detected for lysozyme alone at the two concentrations used (Fig. S1), having an average signal between 160–180 AU.

Transmission electron microscopy (TEM) images captured at the 24 h time point of the aggregation experiment confirmed fibril formation by the aggregated Aβ1-42 sample (Fig. 8D) whilst no fibrils could be detected for the lysozyme samples (Fig. S2). Fibrillar species were formed in the presence of a 1:1 ratio of the Aβ1-42 and lysozyme concentrations; these forms were accompanied by smaller aggregated non-fibrillar species that is approximately 180 AU. Aβ1-42 fibril formation, was bril formed this day was postponed by 7 days, and the cut-off value was not reached until day 21. The control flies demonstrated a normal angle of movement until day 34 (Fig. 4C). The protective effects of lysozyme in the longevity and locomotor assays of the Aβ1-42 expressing flies revealed that lysozyme has a pathomechanistic relevant effect on the reduction of Aβ toxicity.

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fibrillar species (Fig. 8D). In the presence of four-fold higher lysozyme, the aggregated samples were dominated by non-fibrillar structures (Fig. 8D).

Next, the possible co-localization of Aβ1-42 and lysozyme after 24 h of aggregation was investigated. When Aβ1-42 was aggregated in the presence of lysozyme, aggregates were detected by both anti-Aβ and anti-lysozyme antibodies, thereby demonstrating the co-localization of Aβ1-42 and lysozyme. The product formed from the 1:1 ratio of Aβ1-42 and lysozyme appeared as large yellow aggregates where Aβ and lysozyme co-localized; these aggregates were accompanied by some small spheres of Aβ alone (red) and amorphous species of lysozyme alone (green) (Fig. 8E). The product formed from the 1:4 ratio of Aβ and lysozyme appeared as small yellow spheres with almost complete co-localization (Fig. 8E). Using an ELISA assay that specifically detects the aggregated forms of Aβ without detecting monomers (Höltta et al., 2013), we determined that Aβ1-42 samples aggregated for 24 h demonstrated a high level of aggregated Aβ species, whereas Aβ1-42 aggregated in the presence of lysozyme had significantly lower levels of these aggregated Aβ forms (Fig. 8D). These findings support the aggregation kinetics results (Fig. 8A, D and E), which suggested that lysozyme inhibits the fibrillation process of Aβ1-42.

To investigate whether lysozyme could alter the toxic properties of Aβ, the viability of neuroblastoma cells was monitored, 3 days after exposure to Aβ1-42 aggregated with or without lysozyme, using XTT. Aβ1-42 was toxic after 6 h of aggregation, causing cells to shrivel up; however, at 24 h the aggregated Aβ1-42 species showed no toxicity (Fig. 8C). Moreover, lysozyme protected the cells against Aβ1-42 toxicity at 6 h. The TEM images in Fig. 8B show that smaller non-fibrillar species were formed when Aβ1-42 was aggregated in the presence of four-fold more lysozyme for 24 h. No cell toxicity was detected after 24 h of Aβ aggregation with lysozyme, demonstrating that...
that lysozyme (Matarin et al., 2015). Two other reports from Luo et al. demonstrate that lysozyme was overexpressed in Drosophila brains. Levels of Aβ and lysozyme in Drosophila at days 0 and 20 detected by Meso Scale Discovery. A) Amounts of soluble Aβ and insoluble Aβ in flies expressing Aβ in the absence or in the presence of lysozyme expression compared with control flies. B) Amounts of soluble lysozyme in flies expressing lysozyme in the absence and in the presence of Aβ expression compared with control flies. Bars represent means ± SEM, n = 5 (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001).

4. Discussion

Triggering of the innate immune system with the secretion of pro- and anti-inflammatory cytokines is an early event in Alzheimer disease. Lysozyme is an important player in the innate immune system but there has not been any proof of a direct relationship between lysozyme and Alzheimer disease. However, in a recent microarray study from a large screen of more than 12,500 genes, the lysozyme gene was identified to be overexpressed in five mice models of Alzheimer disease (Matarin et al., 2015). Two other reports from Luo et al. demonstrate that lysozyme in vitro binds and prevents the aggregation of Aβ1-40. The present study further reveals the involvement of lysozyme in Alzheimer disease where lysozyme was detected at significantly higher levels in CSF from Alzheimer disease patients compared with controls and was co-localized with Aβ in plaques. We also demonstrate the physiological relevance of lysozyme using a Drosophila model of Aβ1-42 toxicity, where lysozyme overexpression prolonged survival and enhanced the activity of the Aβ1-42 flies. At a mechanistic level, in vitro assays revealed that lysozyme bound to Aβ and influenced the structure of aggregated Aβ1-42, thereby reducing the toxicity of Aβ1-42.

Because lysozyme levels were increased in CSF from patients with Alzheimer disease, we investigated the Alzheimer disease specificity of lysozyme using CSF from patients with increased CSF levels of T-tau but normal Aβ1-42 and P-tau181P values (i.e., patients suffering axonal damage most likely not caused by Alzheimer disease). Elevated levels of T-tau is found after acute damage to the brain such as stroke and subarachnoid hemorrhage, and the levels of these biomarkers correlate with the severity of the brain damage (Hesse et al., 2000). The lysozyme levels did not increase with increasing levels of T-tau, which implies that increased lysozyme levels in the Alzheimer disease CSF is not secondary to general neurodegeneration. The activation of neuro-inflammatory pathways in the brain is emphasized as a major risk factor for the development of Alzheimer disease. Several reports from transgenic mice studies indicated that immune activation might prime the brain for Alzheimer disease pathology, such as Aβ plaques and tau aggregation (Qiao et al., 2001; Sheng et al., 2003; Kitazawa et al., 2005). If the increased lysozyme levels in CSF from Alzheimer disease patients are triggered by early immune activation, the detection of lysozyme in CSF might be useful as a biomarker for Alzheimer disease particularly in its early preclinical phase. We also showed that lysozyme is closely associated with cortical amyloid plaques, therefore the physiological relevance of lysozyme was investigated in a Drosophila model of Aβ1-42 toxicity. Aβ1-42 expressed in the fly CNS resulted in neurological impairments that manifested as effects on the longevity and locomotor assays. The health of Aβ1-expressing flies was remarkably increased when lysozyme was co-expressed with Aβ1; the lifespan was extended to that of normal flies and the locomotor activity was substantially improved. The level of insoluble Aβ was found to be considerably higher in the brains of the Aβ1-42 flies compared with the soluble levels of the these structures do not possess any cytotoxic properties (Fig. 8C). These results suggest that the binding of lysozyme to Aβ1-42 decreases the aggregation propensity, and thereby bypasses the route of formation of toxic Aβ1-42 species.

Fig. 6. Lysozyme counteracts the formation of insoluble Aβ species in Drosophila brains. Levels of Aβ and lysozyme in Drosophila at days 0 and 20 detected by Meso Scale Discovery. A) Amounts of soluble Aβ and insoluble Aβ in flies expressing Aβ in the absence or in the presence of lysozyme expression compared with control flies. B) Amounts of soluble lysozyme in flies expressing lysozyme in the absence and in the presence of Aβ expression compared with control flies. Bars represent means ± SEM, n = 5 (*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001).

Fig. 7. Lysozyme associates with monomeric and fibrillar Aβ. A) Fluorescence intensity of 0.2 μM Alexa-488 labeled lysozyme (donor) in the absence (black line) and in the presence (red line) of 8 μM TAMRA-labeled monomeric Aβ1-42 (acceptor). The decrease of donor fluorescence and increase of acceptor fluorescence indicates fluorescence resonance energy transfer (FRET) between the donor and acceptor due to the association of lysozyme with monomeric Aβ. B) Representative confocal images of 10 μM Aβ1-42 labeled with TAMRA that was aggregated for 24 h and then incubated for 1 h with 40 μM lysozyme labeled with Alexa Fluor 488. The merged image of Aβ1-42 and lysozyme fluorescence demonstrates co-localization of the two proteins. Scale bar = 20 μm.
lysozyme and the insoluble species of the peptide accumulated over time. Thus, it is likely that these insoluble \(\beta\)-amyloid (A\(\beta\)) species or intermediate species formed on the pathway toward insoluble species are cytotoxic and contribute to the reduction in lifespan and locomotor activity observed for the A\(\beta\)-1-42 flies. When A\(\beta\) was co-expressed with lysozyme, the level of insoluble A\(\beta\) species was reduced in both young and aged flies, indicating that the rescue effects in the lysozyme:A\(\beta\) flies are caused by the capacity of lysozyme to counteract the formation of these insoluble A\(\beta\) species, thereby reducing the formation of toxic A\(\beta\) species.

We revealed that lysozyme bound to both monomeric and fibrillar A\(\beta\)-1-42; this result is in agreement with recent studies where lysozyme was demonstrated to bind to monomeric A\(\beta\)-1-40 (Luo et al., 2013, 2014). Additionally, we detected co-localization between lysozyme and A\(\beta\) in aggregates formed both in vivo and in vitro. Our observation that lysozyme reduces the aggregation kinetics of A\(\beta\) is likely caused by the binding of lysozyme to monomeric A\(\beta\) during the initiation of the nucleation phase. In turn, this binding results in a change in the A\(\beta\) aggregation process where fibril formation of A\(\beta\) is prevented and the formation of aggregated non-fibrillar species, composed of both lysozyme and A\(\beta\), is promoted. Studies on cells showed that during the aggregation process of A\(\beta\), cytotoxic aggregated species were formed at 6 h, whereas in the presence of lysozyme no cytotoxic species were formed. This demonstrates that when lysozyme binds to monomeric A\(\beta\), the pool of free monomeric A\(\beta\) peptides that can aggregate and form cytotoxic species is reduced in favor of the formation of non-toxic, non-fibrillar lysozyme-A\(\beta\) species. The formation of these species is likely to occur via a pathway that differs from the A\(\beta\) aggregation pathway towards fibril formation. Luo et al. recently showed that lysozyme also prevents the toxicity of A\(\beta\)-1-40 (Luo et al., 2014). The in vivo step at which lysozyme was able to rescue A\(\beta\) cytoxicity in the fly CNS is likely to be in line with the in vitro effect of lysozyme on the A\(\beta\) aggregation process. By binding of lysozyme to monomeric A\(\beta\) in the fly brain, less A\(\beta\) peptides are able to aggregate into cytotoxic species.

Instead, the lysozyme-bound A\(\beta\) peptides either participate in the formation of other, presumptively non-toxic, aggregates composed of both lysozyme and A\(\beta\) or are degraded, because the degradation of lysozyme and A\(\beta\) seems to be more efficient when the proteins are co-expressed compared with when the proteins are expressed individually (Kumita et al., 2012).

Little is known about the implications of lysozyme involvement in Alzheimer disease. One pathological feature of Alzheimer disease is the failure of the lysosomal clearance mechanisms that manifests as a severe buildup of lysosomal-related compartments in dystrophic neurites (Nixon et al., 2005). Our finding that lysozyme is increased in CSF from Alzheimer disease patients might represent a cellular compensation for decreased lysosomal function in the Alzheimer disease brain (Nixon and Yang, 2011) via the up-regulation of lysosomal proteins such as lysozyme (Gupta et al., 1985). The lysosomal proteins LAMP-1, LAMP-2 and cathepsin D have previously been reported to be increased in CSF from Alzheimer disease patients (Schwagerl et al., 1995; Armstrong et al., 2014). Because neuroinflammation is part of the pathology of most lysosomal storage disorders, the up-regulation of lysosomal proteins might be a general phenomenon in these disorders. A functional loss of the lysosomal system may also affect phagocytosis and recycling in microglial cells, which might in turn lead to microglial activation and increased secretion of lysozyme. Another possibility is that the up-regulation of lysozyme might be directly caused by the accumulation of A\(\beta\) in Alzheimer disease patients, because our study demonstrated that exposure of neuroglia cells to A\(\beta\) caused up-regulated expression and secretion of lysozyme. No secretion of lysozyme was detected from neuroblastoma cells, indicating that the origin of lysozyme in CSF and plaques is astrocytic rather than neuronal.

Our results demonstrating the rescue effects of lysozyme on A\(\beta\) toxicity establish a potential protective role for lysozyme in Alzheimer disease.

**Fig. 8.** Effect of lysozyme on A\(\beta\) aggregation and cytotoxicity. A) Aggregation of 10 \(\mu\)M A\(\beta\) alone or in the presence of 10 \(\mu\)M or 40 \(\mu\)M lysozyme for 24 h probed by ThT. Representative ThT fluorescence curves are shown, \(n = 3\). B) Detection of aggregated A\(\beta\)-1-40 by ELISA of A\(\beta\) aggregated for 24 h alone or with increasing lysozyme concentrations. C) Viability of differentiated SH-SY5Y cells analyzed by XTT after 3 days of exposure to A\(\beta\) aggregated at 10 \(\mu\)M for 6 or 24 h alone or in the presence of 40 \(\mu\)M lysozyme and then diluted 1:10 in cell culture medium. The bars represent the mean \(\pm\) SD, \(n = 4\) (*\(p < 0.05\), **\(p < 0.01\)). D) TEM images of 10 \(\mu\)M A\(\beta\)-1-40 (Luo et al., 2014). The fibrillar and amorphous A\(\beta\) species are discernable in the TEM images. E) TEM images of 10 \(\mu\)M A\(\beta\)-1-40 (Luo et al., 2014). The fibrillar and amorphous A\(\beta\) species are discernable in the TEM images.
Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

The authors thank Damian Crowther for kindly providing α-defensin from the IFelig model and Maria Lindbjer-Andersson for performing the ELISA specific for aggregated α-defensin.

We are grateful for the brain tissue samples from the Sydney Brain Bank at Neuroscience Research Australia and the New South Wales Tissue Resource Centre at the University of Sydney which are supported by the National Health and Medical Research Council of Australia (NHMRC), University of New South Wales, Neuroscience Research Australia, Schizophrenia Research Institute and National Institute of Alcohol Abuse and Alcoholism (NIH (NIAAA) R24AA012725). GMH is a Senior Principal Research Fellow of the National Health and Medical Research Council of Australia (#1079679).

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