Glia in Alzheimer's disease and aging: Molecular mechanisms underlying astrocyte and microglia reactivity

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CHAPTER 2

REACTIVE GLIA SHOW INCREASED IMMUNOPROTEASOME ACTIVITY IN ALZHEIMER’S DISEASE

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Abstract

The proteasome is the major protein degradation system within the cell, comprised of different proteolytic subunits; Amyloid-β is thought to impair its activity in Alzheimer’s disease. Neuroinflammation is a prominent hallmark of Alzheimer’s disease, which may implicate an activation of the immunoproteasome, a specific proteasome variant induced by immune signaling that holds slightly different proteolytic properties than the constitutive proteasome. Using a novel cell-permeable proteasome activity probe, we found that Amyloid-β enhances proteasome activity in glial and neuronal cultures. Additionally, using a subunit specific proteasome activity assay we showed that in the cortex of the APPswePS1dE9 plaque pathology mouse model, immunoproteasome activities were strongly increased together with increased mRNA and protein expression in reactive glia surrounding plaques. Importantly, this elevated activity was confirmed in human post mortem tissue from donors with Alzheimer’s disease. These findings are in contrast with earlier studies, which reported impairment of proteasome activity in human Alzheimer’s disease tissue and mouse models. Targeting the increased immunoproteasome activity with a specific inhibitor resulted in a decreased expression of inflammatory markers in ex-vivo microglia. This may serve as a potential novel approach to modulate sustained neuroinflammation and glial dysfunction associated with Alzheimer’s disease.
INTRODUCTION

One of the major hallmarks of Alzheimer’s disease (AD) is the deposition of extracellular plaques, composed of aggregated amyloid-β (Aβ). Other hallmarks of AD are the presence of intra-neuronal aggregates of hyper-phosphorylated tau [10], activated microglia [4], and astrogliosis [5]. An accumulation of aberrant and ubiquitinated proteins in association with Aβ plaques, tangles and dystrophic neurites is observed in AD brains, and has been postulated to be the result of an impaired ubiquitin proteasome system (UPS) [154]. The UPS is the main protein degradation system within cells; it plays a key role in degrading aberrant proteins and is involved in a variety of vital cellular processes, such as degrading short-lived proteins, and generation of peptides for antigen presentation on MHC class I molecules. The proteolytic component of the UPS is the proteasome (PS). The constitutive proteasome (cPS) is composed of a 20S core including three proteolytic subunits β1, β2, and β5, each possessing a different enzymatic activity; caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L), respectively [154, 155]. Pro-inflammatory mediators, such as interferon gamma (IFNγ), induce the formation of a PS variant termed the immunoproteasome (iPS). The iPS has distinct proteolytic subunits (β1i, β2i, and β5i) making it better equipped for cleavage of peptides for antigen presentation which is believed to be its main function [91, 103]. Recent studies show that the iPS also has immune regulatory functions, involved in the modulation of innate immune signaling [95].

Several studies have provided evidence for an impaired proteasome activity in AD but these assays did not discriminate between iPS- and cPS-mediated activities. Assays in homogenates from AD affected brain areas showed impaired CT-L and C-L PS activities [116, 120, 121]. Inhibition of PS activity has also been shown in several AD mouse models [119, 122, 123]. However, there is also data providing evidence that AD is linked to an increased PS activity. Cell lysates of Aβ treated primary rat astrocytes showed an initial impairment of CT-L activity, followed by an increase at later time points [156]. Increased iPS activity may also be expected as a result of the plaque-related inflammatory signaling in AD [6, 65, 125]. Moreover, our group has previously found that inhibition of the proteasome leads to a decrease
in expression of the astrocyte reactivity markers, GFAP and vimentin [157]. This is in sharp contrast to the high levels of GFAP expression in reactive astrocytes surrounding plaques [5, 6]. We concluded that the evidence for a decreased PS activity in AD tissue presented so far is not entirely conclusive and that an increased PS would actually be more in line of our expectation. Studies up to now have mainly focused on the effect of Aβ on PS activity in neurons. However, reactive astrocytes and activated microglia in the inflammatory environment in AD brains may respond differently to extracellular Aβ and plaques. Here, we studied the effects of Aβ on proteasome activity on different cell types in vitro and the alterations of PS in a mouse model for AD and in post mortem human AD tissue.

MATERIALS AND METHODS

Cell culture, treatment and differentiation

Mouse astrocytoma Ink/Arf<sup>−/−</sup> cells were cultured in DMEM/F-12 medium supplemented with 10 % FBS and 1% penicillin/streptomycin (P/S), the mouse immortalized microglia N9 was cultured in DMEM glutamax and 5% FBS, 1% P/S and 1% sodium pyruvate and the mouse neuroblastoma cell line N2A were cultured in DMEM glutamax and 10% FBS, 1% P/S and differentiated by serum starvation (0.5% FBS) for 3 days. All cells were kept in a 37°C/ 5% CO<sub>2</sub> incubator. Cells were treated with Aβ1–42 fibrils or oligomers 1.0 μM, 0.1 μM. For proteasome inhibition a dilution series of Epoxomicin (Santa Cruz Biotechnology) was used. All culture media were purchased from Invitrogen. Primary cells used for amyloid beta treatment were isolated as described previously [352] from p3 mouse cortex. After dissociation, cells were allowed to adhere to the uncoated plastic bottom of a cell culture flask, after 2h the supernatant was removed, adherent cells containing a mix of astrocytes and microglia were allowed to grow for 14 days prior usage for Aβ treatment.

Mice

APPwePS1dE9 double-transgenic mice [158] were studied, wild type (WT), sex-matched, littermate pairs were used as controls. For analysis of UPS functioning in
In vivo, the UbG76VGFP/2 mice [159] were crossed with the APPswePS1dE9 mice to generate AD mice with a GFP UPS reporter. All animals were housed under standard conditions. Experimental procedures were approved by the ethical committee for animal care and use of the Royal Netherlands Academy of Arts and Sciences.

For immunofluorescence, groups of AD and WT animals were studied at the age of 3, 6, 9, 12, 15, and 18 months. For qPCR assays, RNA isolated from the cortex of 5-8 AD animals was compared with RNA isolated from 8-11 WT mice of the same age groups. For proteasome activity (PS activity) assays, frozen cortices from the same mice were used. For the qPCR, immunohistochemistry, and PS activity experiments equal numbers of male and female mice were used. As no significant differences were noted, males and females were pooled.

**Immunofluorescence and immunohistochemical procedures.**

Coronal cryosections (10 μm) of mouse cortical tissue and human post-mortem hippocampal/entorhinal cortex tissue were post-fixed in 4% paraformaldehyde in PBS, pH 7.0, at room temperature. Sections were blocked with 0.05M PB supplemented with 10% normal donkey serum and 0.4% Triton X-100, incubated overnight with the primary antibodies diluted in 3% normal donkey serum and 0.4% Triton X-100 in PB at room temperature. For antibodies see Supplemental Table 2. Negative controls were included by omitting the primary antibody, but these never yielded staining patterns. For mouse tissue, fluorescence visualization was used with Cy3- or Alexa 488-conjugated secondary antibodies. Sections were washed and cover-slipped in Vectashield, including DAPI as a nuclear dye (Vector Laboratories) and viewed with a Leica DMRE fluorescence microscope (Leica). For immunohistochemistry on human tissue, 3,3′-Diaminobenzidine (DAB) and Alkaline phosphatase (AP) double staining was done using biotinylated- and AP labeled secondary antibodies. Prior DAB development an amplification step was performed, using avidin-biotin complex (1:800 in PB; Vector laboratories), followed by washing and incubation with DAB solution (Sigma-Aldrich). For development of AP the Vector® Blue Substrate Kit (Vector laboratories) was used according to manufacturers’ instructions. Images of DAB/ AP double labeling with pseudocolors were taken on an AxioVert microscope with Neoplanfluor objectives using the multispectral imaging system camera.
CRI Nuance FX (Quorum Technologies Inc) and the Nuance software (Cambridge Research and Instrumentation inc). Immunohistochemistry was performed on tissue from four different donors. Adobe Photoshop was used to arrange the TIFF files for presentation. For quantification of the BrdU/β5i co-localization and Iba1 stain in the mouse tissue, cell counts were made bilaterally in the neocortex. The cell counts were averaged reflecting the number of cells observed in a single 10 μm section per unilateral side (hemisphere), n: 4-6 mice as in [6].

**Amyloid beta peptide preparations**

Preparation of oligomeric Aβ (oAβ) and fibrillar Aβ (fAβ) was done as described previous [160]. Aβ1–42 peptides (Anaspec) were dissolved in HFIP (1,1,1,3,3,3-hexafluoroisopropanol (Sigma) at a final concentration of 1mg/ml, followed by speedvac. The peptide film was dissolved in DMSO and sonicated. For Aβ1–42 oligomers, the peptide/DMSO mix was added to ice-cold 25mM PBS, pH 7.4 during vortexing, followed by incubation at 4°C for 24h followed by centrifugation. The supernatant containing the oligomeric fraction was snap frozen and stored at -80° C. For Aβ1–42 fibrils; the peptide/DMSO mixture was added to 10mM HCl, vortexed, incubated at 37°C for 48h, and centrifuged. The pellet containing the fibrils was dissolved in 10mM HCl/5% DMSO, snap frozen, and stored at – 80° C. Beta sheet content was measured by Thioflavin T assay (Sigma) and protein concentrations by Bradford (Bio-Rad) according to manufacturer’s protocol. Aggregation was checked on western blot using 12% Bis/Tris gels (Invitrogen) blotted on nitrocellulose membrane and stained with 6E10 antibody.

**PS activity in living cells**

Cell lines were treated with Aβ1–42 fibrils or oligomers in concentrations of 0.1 µM or 1.0 µM for 72h. After washing in (FBS free) DMEM, the cells were incubated with 200nM Bodipy-tagged, cell-permeable, proteasome probe, Bodipy-FL-Ahx3L3VS [161] for 2h. Cells were trypsinized and washed in DMEM followed by centrifugation 200 x g, 5 min at 4°C and resuspended PBS/ 1% BSA, 7AAD (BD biosciences) viability dye was added, followed by analysis by flow cytometer using a BD FACS Calibur (BD Biosciences). For cell lines the geometric mean of probe intensity from 20,000 viable cells was used as measurement for average sample PS activity.
A co-culture of primary astrocytes and microglia were treated with 1.0 µM oAβ or fAβ for 72h and analyzed using the proteasome probe as described above. The microglia were labeled with an APC conjugated anti-CD11b antibody (eBioscience) enabled us to separate astrocytes from microglia by flow cytometric analysis. A total of 6000-20,000 primary glia cells were analyzed per condition. The geometric mean of the probe intensity was assessed in the CD11b+ microglia and the CD11b− astrocytes separately. For all cell types and cell lines, Aβ-treated samples were compared to mock treated control samples in each assay.

**Tissue and cell homogenization**

**Tissue:** Snap frozen left cortices from APPswePS1dE9 mice and WT littermates were homogenized in a pair-wise manner in homogenization buffer (50 mM Hepes, 250 mM sucrose, 5 mM MgCl₂, 0.5 mM DTT and 40 mM KCl pH 7.4). Homogenates were prepared using an ultra turrax, followed by centrifugation at 4 °C as follows: 1,300 x g for 10 min; 10,000 x g for 10 min and 13,000 x g for 25 min, transferring the supernatant in each step. The protein concentration of the final supernatant was determined with the BCA protein assay kit (Thermo Fisher) by using a flurometric 96-well reader (Varioskan Flash reader, Thermo Fisher Scientific).

**Cells:** Cells were cultured in 6-well plates and treated with Aβ1–42 oligomers or fibrils in concentrations of 0.1 µM, 1.0 µM or mock as control for 72h. Cells were trypsinized, washed, and pelleted at 200 x g, 5 min at 4°C. The pellets were resuspended in 200 μl homogenization buffer and incubated with Digitonin 0.025% (Invitrogen) on ice for 5 min. The cytosolic cell fraction was “squeezed out” by centrifugation at 20,000 x g for 15 min at 4 °C. The cytosolic protein content was measured by Bradford assay and 20 µg of protein was used for analysis of PS activity using AMC conjugated reported peptides. PS activity assay in brain homogenates using AMC conjugated peptides

The 7-amino-4-methyl coumarine (AMC) conjugated activity-specific peptides were used to assess the different proteasome subunit activities, 50 µM Boc-LRR-AMC for the trypsin-like activity, 50 µM Suc-LLVY-AMC for chymotrypsin-like activity (both BostonBiochem), and 25 µM Ac-nLPnLD-AMC (Bachem AG) to measure the caspase-like activity. 20 µg of protein per reaction was diluted in assay buffer (50 mM Hepes, 20 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10 µM ATP (pH 7.4)).
and incubated with the peptides. To control for non-proteasomal-mediated cleavage of substrates, a proteasome inhibitor was used in concentrations that give a complete block of the PS activity. Values from inhibited samples were subtracted from non-inhibited samples to assess proteasome activity. For the caspase-like and chymotrypsin-like activity, 15 μM and 5 μM Epoxomicin were used, respectively and for the trypsin-like activity 40 μM MG132 was used. Samples were incubated with inhibitors at room temperature for 30 min. Activity was assessed by continuous fluorescent measurement of the released AMC at 37°C for 1 hour, using a fluorometric plate reader (Varioskan Flash reader). A regression analysis was done on the most linear part of the time/fluorescence curve. The slope of the trend line was taken as a measurement for the proteolytic activity. Littermate AD and WT couples were assayed at the same time. Difference was analyzed using paired t-tests on couples.

Proteasome Constitutive-Immuno Subunit ELISA assay (ProCISE)
The ProCISE assay was used to examine the subunit-specific proteasome activities of the constitutive (β5, β1, β2) and immunoproteasome (β5i, β1i, β2i) active sites in tissue samples of mouse cortices from AD and WT mice and in hippocampal / entorhinal cortex tissue samples of human donors. This assay was performed as previously described [162] with the exception of the use of a different β1 antibody for mouse tissue (Santa Cruz Biotechnology, Cat. No. sc-67345). Tissue samples were dissolved in lysis buffer (20 mM Tris-HCl pH 8.0, 5 mM EDTA) to 1 mg/ml, then incubated with a proteasome active site probe (PABP; 5 μM) for 2 hours at 25°C. Samples were denatured in 6 M guanidine hydrochloride (Fisher Scientific) and subunits bound to PABP were captured with streptavidin-conjugated sepharose beads (GE Healthcare) in 96-well 0.65 µm porous filter plates (Millipore). Individual subunits were probed with subunit-specific primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). The SuperSignal ELISA Pico chemiluminescent substrate kit (Pierce) was utilized to generate luminescent signal associated with HRP binding. Luminescence after 5 minutes of signal development was monitored on a plate reader (Tecan Safire). For mouse samples, subunit activity was expressed as relative luminescence units normalized to protein quantity. For human samples, subunit content was expressed as nanograms of subunit per.
microgram of total protein, as calculated from a standard curve of human purified constitutive or immunoproteasome, assayed simultaneously with the samples.

**Laser dissection microscopy**

Sections from snap frozen brains from WT and AD mice of 6, 9 and 12 months were mounted onto glass slides with PENfoil membrane. Slides were washed for 5 min in 70% EtOH and incubated in BTA-1 (2 - (4'' - (methylamino) phenyl) benzothiazole) in diluted in 75% EtOH for 7 min, to visualize Aβ plaques. The slides were washed in 70% EtOH, dried and subjected to LMPC. The LDM platform used in this study is the PALM Microbeam system and PALM Software (PALM Robo 3.5). Forty plaques and non-plaque regions were isolated from the somatosensory cortex of AD and WT mice. Dissected areas had a diameter about twice the stained plaque size (Fig 2A, B). RNA was isolated from the dissected samples as below. Expression was normalized on GAPDH.

**Isolation of primary cells for Aβ treatment and proteasome inhibition**

For ex-vivo PS inhibition of AD and WT microglia: AD and WT mice were anesthetized and perfused with HBSS (-Ca/Mg) (Invitrogen). The cortices were dissected and subjected to mechanical dissociation using Tissue Chopper, followed by enzymatic dissociation using Papain (Worthington) at a final concentration of 8 U/ml together with DNase I at 80 Kunitz/ml (Sigma) at 37°C incubator for 50 min and thereafter pelleted. The pellet was triturated to single cell suspension in MEM 1% BSA and filtered through a 20 μm single cell strainer (BD Biosciences) followed by a separation step over an isotonic (90%) percoll (Amersham Biosciences) at 200 x g for 15 min at 4°C. Top phase were discarded, the other layers were collected and resuspended in MEM 1% BSA in five times the volume followed by centrifugation for 10 min at 4°C. Cells were stained with APC labeled CD11b antibody and Fc-receptor block (CD16/32) (all eBioscience), in PBS (0.5% BSA) at 4°C for 30 min. The cells were washed and resuspended in PBS/BSA and cell viability staining 7AAD (BD bioscience). Using BD FACS Aria I (BD bioscience), CD11b+ microglia were sorted after gating away the dead cell population on 7AAD signal. Sorted microglia were
resuspended in DMEM/F-10, 5% FCS, 1% P/S, 20-30,000 cells were inhibited with either 200 nM of the β5i inhibitor ONX-0914 (previously named PR-957) or 125 nM of the PR-825 β5 inhibitor or vehicle for 1h at 37°C, 5% CO₂. Activity specific concentrations of the inhibitors were based on previous studies [128, 130]. Cells were washed and plated, and stimulated with LPS 1µg/ml (from Escherichia coli 026:B6, L2654, Sigma) for 14h at 37°C, 5% CO₂. Cells were washed and TRIzol was added for RNA isolation (see section below for RNA isolation procedure).

Quantitative polymerase chain reaction (qPCR) assays
For gene expression analysis of cortex, the right hemisphere was used for RNA isolation. For human samples 20x 10 µm sections from snap frozen post mortem hippocampi obtained from the Netherlands Brain Bank were used (For donor data see Supplemental Table 1). Tissue was homogenized in Trizol using an Ultra Turrax. Cells and LDM material were allowed to incubate in Trizol for 5 minutes followed by thorough mixing/vortexing. RNA was isolated by precipitation overnight in isopropanol with addition of 20 µg glycogen. Total RNA was DNaseI treated and used as a template to generate cDNA following the manufacturer’s instructions (Quantitect-Qiagen). Diluted cDNA served as a template in real-time quantitative PCR assays (SYBR® Green PCR Master Mix; Applied Biosystems). Gene expression on mice cortex samples, and isolated microglia samples were normalized using the geometric mean of GAPDH, HPRT and Actin expression. Expression levels of human AD samples were normalized against a selection of 10 reference genes (GAPDH, ACTB, PPIA, UBE2D2, EEF1A, RPS27A, AARS, XPNPEP1, RPLP0, IPO8) [163, 164] based on a geNorm analysis [165]. The normalization factor was the geometric mean of the 10 reference genes. Samples with a RIN value below 5.0 were excluded from analysis based on a poor correlation with the normalization factor. For primers see Supplemental Table 3.

Statistics
Statistical analyses were done in SPSS, PAWS statistics, 18.0 (Predictive Analytics SoftWare) and GraphPad Prism 5 (GraphPad Software Inc). Data was tested for
normal distribution using Shapiro Wilk test for normality. For single comparisons
between age-matched AD and WT groups, or between Aβ and mock treated sam-
pies, significance was tested using a Student’s t test or a Mann-Whitney U test. For
multiple comparisons between groups the Kruskal-Wallis test with Dunn’s multiple
comparison was used. For analysis of the human ProCISE assay, an ANOVA was
performed using Games-Howell post-Hoc to test for differences between pathology
stages. For correlations Pearson’s correlation was used for normally distributed data,
in other cases the Spearman’s Rho non-parametric correlation was used. In all cas-
es statistical significance was asserted for p < 0.05.

Results
Aβ increases the PS activity in cultured astrocytes, microglia, and neurons
A decrease in PS activity has been reported in brain homogenates of AD patients
[116, 120, 121], AD mouse models [119, 122, 123], and in lysates of Aβ treated neu-
rons in vitro [122]. Here we investigated the effects of Aβ 1-42 fibrils (fAβ) and oligo-
mers (oAβ) on PS activity in viable astrocytes, microglia, and a neuronal cell lines.
Cells were treated with 0.1 µM or 1.0 µM of either oAβ or fAβ (Supplemental Fig.
1A) for 72h. The PS activity was determined in living cells using a cell-permeable
fluorescent proteasome specific probe [157, 161] analyzed by flow cytometry (Fig.
1A). The probe was shown to be PS activity-specific since the fluorescent signal was
reduced by adding increasing concentrations of the specific PS inhibitor Epoxomicin
(Fig. 1B, Supplemental Fig.1B-C) [161]. Astrocytes (Fig. 1C), neurons (Fig. 1D), and
microglia (Fig. 1E) showed an increased PS activity in response to treatment with
1µM fAβ or 1µM oAβ for 72h (Fig. 1C-E). In microglia and neurons, fAβ or oAβ at a
concentration of 0.1 µM had no significant effect on PS activity, while 0.1µM fAβ in-
creased PS activity in astrocytes. In concordance with the results above, astrocytes
treated with 1.0 µM fAβ for 72h showed enhanced proteolysis of the classical Ami-
no-4-methyl coumarin (AMC)-conjugated peptides for CT-L and C-L activities of the
PS. Astrocytes treated with oAβ for 72h, showed a near significant increase of both
peptides (Supplemental Fig. 1D-E). No significant differences in gene expression of
cPS and iPS subunits between Aβ or mock treated cells were found (Supplemental
Fig. 2 A-B). To confirm the data from the glial cell lines, mixed primary mouse glial cells were treated with 1.0 µM fAβ or 1.0 µM oAβ for 72h followed by proteasome activity assessment using the cell permeable PS activity probe. The PS activity of microglia and astrocytes was separated based on positive labeling of the microglia using CD11b+ antibodies. (see Supplemental Fig. 3A-C” for culture staining and flow cytometry plots). The CD11b+ astrocyte population showed a near significant increase in PS activity by 15% upon treatment with 1.0 µM fAβ while no difference was observed with 1.0 µM oAβ (Fig. 1F and Supplemental Fig. 3C”). The CD11b+ microglia population, which made up on average 8.5% of the total cell population, showed a significant 98% increase of PS activity upon treatment with 1.0 µM fAβ, while 1.0 µM oAβ treatment showed no effect (Fig. 1G and Supplemental Fig. 3C’). Innate immune activation is a reported feature in AD [166] and lipopolysaccharide (LPS) is a potent stimulant of innate immune signaling [167]. LPS stimulation of the microglia cell line for 24h resulted in a two-fold increase of PS activity compared to untreated cells (Fig. 1E), as well as a significant increase in mRNA expression of all cPS and iPS proteolytic β subunits (Fig. 1H). The fold increase of the iPS subunits was larger than the fold increase of the proteolytic cPS subunits (Fig. 1H). Taken together, these data show that especially fAβ but also oAβ increase, rather than inhibit the PS activity in living astrocytes, microglia, and neurons. Additionally, a strong innate immune activation by LPS elevated the PS activity in microglia through increased transcription mainly of the iPS subunits. These data suggest that in AD brain PS activity may not only be increased as a result of a direct effect of Aβ, but also as a consequence of the inflammatory conditions caused by Aβ plaque deposition.

**Plaques associated glia show an increase in the expression of iPS subunits**

Several genes involved in UPS-mediated protein degradation and markers for glial cell reactivity were analyzed by qPCR to determine differential expression in AD and WT mice of different ages. Expression fold changes (FC) are shown in Table 1. At 9 months and older, transcript levels of β5i (Psmb8) were significantly increased, and from 12 months and older for β1i (Psmb9), with the highest FC of 2.11 for β1i (Psmb9) at 18 months and 2.93 for β5i (Psmb8) at 12 months (Table 1). No differences were found in transcript levels of the other proteolytic and non-proteolytic
Figure 1. Aβ treatment increases the proteasome activity in living astrocytes, microglia and neurons in vitro. (A) Histograms showing the fluorescence of the cell-permeable proteasome activity probe as measured by flow cytometry. The x-axis shows the probe fluorescence in (FL-1). Aβ fibrils (1.0 μM) increased the fluorescence signal, indicating more probe bound and an increase in PS activity (solid black line) compared to mock treated controls (solid gray line). Inhibition of the proteasome using Epoxomicin showed strong reduction in fluorescence (dotted black line). (B) Treatment with increasing concentrations of the proteasome inhibitor Epoxomicin (2.5-100nM) for 2h reduced the fluorescence of the probe in a concentration dependent manner in Ink4a/Arf<sup>−/−</sup> astrocytic cells. Proteasome activity measured with the activity probe in (C) Ink4a/Arf<sup>−/−</sup>(astrocytoma), (D) N2A (differentiated neuroblastoma), and (E) N9 (immortalized microglia) treated with oAβ1-42 and fAβ1-42 in concentrations of 0.1 μM and 1.0 μM for 72h. (C-E) Increased PS activity was observed in all three cell types after stimulation with fAβ and oAβ at 1.0 μM concentration. Only astrocytes displayed increased PS activity to 0.1 μM fAβ. (E) LPS was used as a positive immune stimulatory control (1μg/ml) and enhanced the PS activity in microglia after 24h, compared to untreated control. (F) Primary astrocytes and (G) primary microglia treated with fAβ and oAβ at 1.0 μM concentration for 72h. Primary microglia showed increased PS activity after stimulation with 1.0 μM oAβ compared to mock treated controls (gray dotted line). (H) Microglia (N9) stimulated with LPS (1μg/ml) for 24h showed an induction of mRNA expression of all proteolytic PS subunits compared to untreated controls (gray dotted line). The induction of the iPS expression was higher than the cPS expression. (B-G) Bars represent average % of PS activity compared to
Figure 2. Microglia and astrocytes showed an increase in immunoproteasome expression around Aβ plaques in AD mice. To localize the expression of iPS and astrocyte reactivity marker, plaque areas stained with BTA-1 were dissected using LDM (A-B) together with non-plaque areas from the same tissue and compared with LDM dissected WT areas. (C) Gfap was increased in AD, both in non-plaque and plaque areas compared to WT, and in plaque areas compared non-plaque areas. β1i (Psmb9) and β5i (Psmb8) expression was upregulated in AD plaque areas compared to WT areas, and in plaque areas compared to non-plaque areas. Expression of β2i (Psmb10) showed no differential expression between any of the groups. In (C) bars represent normalized mRNA expression in fold over WT, n; WT=20, AD=11-12. * compared to WT, # compared to non-plaque. Significance was tested using Mann-Whitney U test, error bars show SEM. (*p<0.05, **p<0.01, ***p<0.001; # p<0.5, #p<0.01, ### p<0.001).

(D) Immunostaining for the iPS subunit β5i revealed an intense staining around plaques in 9 months old AD mouse and showed a clear overlap with the Iba1-positive microglia (E) as shown in the merged picture (F). Activated amoeboid shaped microglia showed a more cytosolic β5i staining (insert x) compared to the resting more ramified microglia (insert x). (G)
β-subunits. Amongst the α-subunits, all but Psma4 showed unchanged expression; Psma4 expression was decreased with 0.63 fold to WT at 18 months (Table 1). In the same set of cDNA samples, we previously demonstrated increased transcript levels of Gfap, Cd11b, and Cx3cr1 as indicators for reactive gliosis and microglia activation [6]. To obtain evidence that the increase of iPS transcript levels in AD mice was associated with Aβ plaques, cortical plaque and non-plaque areas were dissected using laser microdissection from brain sections of AD mice (Fig. 2A-B). Expression levels of iPS subunits and Gfap in microdissected plaque areas were compared to non-plaque areas, and to areas dissected from age-matched WT mice (Fig. 2C). Plaque areas showed increased mRNA expression levels of β5i (FC = 11.3) compared to WT areas and to AD non-plaque areas (FC= 5.0) (Fig. 2C). The expression level of β1i was increased with a FC of 7.5 compared to WT and a FC of 4.7 compared to non-plaque areas. No change in β2i expression was observed in any of the areas (Fig. 2C). In the same samples Gfap levels were significantly increased in plaque areas, which show that the increased expression of iPS subunits is confined to the astrogliotic area surrounding the Aβ plaques.

iPS subunits are present in astrocytes and proliferative plaque associated microglia

To examine whether the increase of transcript levels leads to an increase in protein levels, and to localize the expression to specific cell types, immunostainings for the iPS subunits were performed in the cortex of AD mice. Staining for β5i was increased around plaques and overlapped with the microglia-specific marker Iba1 (Fig. 2D-F). In non-activated, ramified, microglia, a weak β5i staining was detected that was mostly restricted to the nucleus (insert x in Fig. 2F), whereas in activated amoeboid microglia, β5i labeling had a more widespread cytosolic distribution (insert y in Fig. 2F). A weak nuclear β5i stain was also observed in reactive astrocytes around plaques (Supplemental Fig. 4C-C’). Double staining with earlier injected

Staining for β1i was present in close proximity of plaques and co-localized with GFAP (H) as shown in the merged image (I). (J) β2i immunostaining was found to be only slightly increased around plaques, partly co-localizing with reactive astrocytes (GFAP) (K-L). Scale bar 20 μm in image D-L. Images are representative of at least two independent experiments.
BrdU [6] and β5i showed that many β5i positive microglia were proliferating, visualized by BrdU incorporation in cells double positive for β5i and Iba1 (Supplemental Fig. 4D-D’). Increased numbers of β5i and BrdU double positive cells were found in AD mice (Supplemental Fig. 4E). The expression of β1i was also found increased around plaques and co-localized with GFAP (Fig. 2G-I). Some β1i staining showed co-localization with the neuronal marker NeuN (Supplemental Fig. 4A-A’). Immunostaining for β2i displayed a similar staining pattern as for β1i, albeit less clear (Fig. 2J-L; Supplemental Fig. 4B-B’). None of the activated glial cells surrounding plaques showed accumulation of ubiquitin, while dystrophic neurites visualized with an antibody against the c-terminus of APP were positive for ubiquitin (Supplemental Fig. 5).

Expression of proteasome subunits in Alzheimer’s tissue

The iPS expression in AD mice was validated in post mortem tissue from hippocampi of controls and AD patients. Gene expression levels of the proteolytic iPS and cPS subunits were analyzed in hippocampi from controls and AD patients of different disease stages based on the Braak scores of tau [168] and amyloid pathology [169] (see Supplemental Table 1 for clinicopathological information) (Table 2 A-B). Expression analysis of the cPS subunits showed an overall decrease of β5 (PSMB5) expression at all Braak stages reaching statistical significance at Braak 2, 5, and 6 with a 38%, 36%, and 38% reduction, respectively. β5 expression was also reduced when grouping on amyloid score and differed significantly at amyloid score B and C compared to amyloid O by 36% and 35 %, respectively. The expression of the other cPS subunits did not show significant changes based on either tau or amyloid scores (Table 2A-B). Of the iPS subunits, the β5i (PSMB8) was significantly increased by 42% at Braak 5, compared to the Braak 0 controls, and by 40% at amyloid score C compared to amyloid O. The β2i expression was increased at Braak 2 by 33%

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<td>The table show mean fold changes (FC) ± SEM of normalized gene expression quantified by RT-qPCR. The different proteasome subunits, non-proteolytic α subunits and the proteolytic β subunits, together with astrocyte and microglia markers were analyzed and compared between the APPswePS1dE9 AD mouse and littermate WT controls. Significance was tested using Kruskal-Wallis test with Dunn’s multiple comparison test, * p&lt;0.05, **p&lt;0.001, ***p&lt;0.001. Gene aliases shown in brackets</td>
</tr>
</tbody>
</table>
Table 1 Expression fold change of proteasome subunits in AD mice compared to age matched WT mice

<table>
<thead>
<tr>
<th></th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
<th>12 months</th>
<th>15 months</th>
<th>18 months</th>
</tr>
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<tbody>
<tr>
<td><strong>α subunits</strong></td>
<td></td>
<td></td>
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<tr>
<td>Psma1</td>
<td>1.14 ± 0.18</td>
<td>1.12 ± 0.13</td>
<td>0.91 ± 0.09</td>
<td>1.11 ± 0.21</td>
<td>0.88 ± 0.09</td>
<td>0.77 ± 0.13</td>
</tr>
<tr>
<td>Psma2</td>
<td>0.99 ± 0.11</td>
<td>1.15 ± 0.06</td>
<td>1.15 ± 0.17</td>
<td>0.97 ± 0.07</td>
<td>0.93 ± 0.07</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>Psma3</td>
<td>0.67 ± 0.16</td>
<td>1.03 ± 0.12</td>
<td>0.90 ± 0.18</td>
<td>1.10 ± 0.08</td>
<td>0.91 ± 0.15</td>
<td>1.25 ± 0.09</td>
</tr>
<tr>
<td>Psma4</td>
<td>0.83 ± 0.08</td>
<td>1.06 ± 0.07</td>
<td>0.88 ± 0.18</td>
<td>0.98 ± 0.08</td>
<td>1.01 ± 0.12</td>
<td>0.63 ± 0.06*</td>
</tr>
<tr>
<td>Psma5</td>
<td>1.66 ± 0.51</td>
<td>1.89 ± 0.88</td>
<td>1.38 ± 0.40</td>
<td>1.03 ± 0.20</td>
<td>1.19 ± 0.25</td>
<td>1.26 ± 0.52</td>
</tr>
<tr>
<td>Psma6</td>
<td>0.99 ± 0.09</td>
<td>1.24 ± 0.10</td>
<td>0.98 ± 0.15</td>
<td>0.96 ± 0.08</td>
<td>1.11 ± 0.12</td>
<td>0.83 ± 0.09</td>
</tr>
<tr>
<td>Psma7</td>
<td>1.60 ± 0.45</td>
<td>0.83 ± 0.25</td>
<td>1.13 ± 0.19</td>
<td>1.24 ± 0.43</td>
<td>0.90 ± 0.34</td>
<td>1.02 ± 0.49</td>
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<tr>
<td><strong>Non-proteolytic β subunits</strong></td>
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<tr>
<td>Psmb1</td>
<td>1.02 ± 0.11</td>
<td>1.16 ± 0.08</td>
<td>0.86 ± 0.13</td>
<td>1.10 ± 0.11</td>
<td>0.89 ± 0.08</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>Psmb2</td>
<td>1.00 ± 0.08</td>
<td>1.09 ± 0.09</td>
<td>1.19 ± 0.23</td>
<td>0.85 ± 0.15</td>
<td>0.90 ± 0.22</td>
<td>0.76 ± 0.10</td>
</tr>
<tr>
<td>Psma3</td>
<td>1.21 ± 0.11</td>
<td>1.04 ± 0.10</td>
<td>0.90 ± 0.17</td>
<td>1.06 ± 0.10</td>
<td>1.00 ± 0.11</td>
<td>0.84 ± 0.10</td>
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<tr>
<td>Psmb4</td>
<td>1.08 ± 0.07</td>
<td>1.08 ± 0.08</td>
<td>1.27 ± 0.23</td>
<td>0.94 ± 0.10</td>
<td>1.04 ± 0.08</td>
<td>0.87 ± 0.04</td>
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<tr>
<td><strong>Proteolytic constitutive β subunits</strong></td>
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<tr>
<td>Psmb5 (β5)</td>
<td>1.17 ± 0.11</td>
<td>0.99 ± 0.14</td>
<td>1.04 ± 0.14</td>
<td>0.82 ± 0.05</td>
<td>0.95 ± 0.06</td>
<td>1.15 ± 0.13</td>
</tr>
<tr>
<td>Psmb6 (β1)</td>
<td>1.34 ± 0.17</td>
<td>1.01 ± 0.10</td>
<td>1.28 ± 0.32</td>
<td>1.11 ± 0.23</td>
<td>0.84 ± 0.10</td>
<td>1.02 ± 0.20</td>
</tr>
<tr>
<td>Psmb7 (β2)</td>
<td>0.92 ± 0.11</td>
<td>1.14 ± 0.10</td>
<td>1.12 ± 0.23</td>
<td>1.10 ± 0.11</td>
<td>0.97 ± 0.05</td>
<td>0.78 ± 0.13</td>
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<tr>
<td><strong>Proteolytic immunoproteasome β subunits</strong></td>
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</tr>
<tr>
<td>Psmb8 (β5i)</td>
<td>0.79 ± 0.14</td>
<td>1.37 ± 0.20</td>
<td>2.07 ± 0.29*</td>
<td>2.93 ± 0.29**</td>
<td>2.76 ± 0.42***</td>
<td>2.08 ± 0.21**</td>
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<tr>
<td>Psmb9 (β1i)</td>
<td>0.83 ± 0.10</td>
<td>1.00 ± 0.13</td>
<td>1.34 ± 0.24</td>
<td>1.65 ± 0.18**</td>
<td>1.83 ± 0.15***</td>
<td>2.11 ± 0.34**</td>
</tr>
<tr>
<td>Psmb10 (β2i)</td>
<td>1.47 ± 0.33</td>
<td>1.09 ± 0.32</td>
<td>1.20 ± 0.23</td>
<td>1.25 ± 0.15</td>
<td>1.23 ± 0.17</td>
<td>1.04 ± 0.35</td>
</tr>
<tr>
<td><strong>Proteasome modulators</strong></td>
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</tr>
<tr>
<td>Psme1 (PA28a)</td>
<td>1.04 ± 0.08</td>
<td>1.01 ± 0.11</td>
<td>1.20 ± 0.16</td>
<td>1.28 ± 0.15</td>
<td>1.20 ± 0.14</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>Psme4 (PA200)</td>
<td>0.62 ± 0.07</td>
<td>0.95 ± 0.08</td>
<td>1.22 ± 0.26</td>
<td>0.96 ± 0.09</td>
<td>1.10 ± 0.03</td>
<td>0.78 ± 0.11</td>
</tr>
<tr>
<td>Psmt1 (P31)</td>
<td>1.07 ± 0.12</td>
<td>1.11 ± 0.10</td>
<td>1.57 ± 0.37</td>
<td>0.96 ± 0.08</td>
<td>1.03 ± 0.04</td>
<td>0.97 ± 0.20</td>
</tr>
<tr>
<td>Pomp</td>
<td>0.89 ± 0.09</td>
<td>0.99 ± 0.09</td>
<td>1.13 ± 0.13</td>
<td>0.97 ± 0.09</td>
<td>1.01 ± 0.17</td>
<td>0.79 ± 0.07</td>
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<tr>
<td><strong>Glial cell markers</strong></td>
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<tr>
<td>Gfap</td>
<td>1.58 ± 0.41</td>
<td>2.39 ± 0.70</td>
<td>4.88 ± 1.00***</td>
<td>5.74 ± 0.69***</td>
<td>6.23 ± 0.82***</td>
<td>5.80 ± 0.96***</td>
</tr>
<tr>
<td>Itgam (Cd11b)</td>
<td>1.00 ± 0.13</td>
<td>1.31 ± 0.23</td>
<td>2.52 ± 0.82*</td>
<td>1.88 ± 0.25**</td>
<td>2.25 ± 0.25***</td>
<td>1.86 ± 0.28**</td>
</tr>
<tr>
<td>Cx3cr1</td>
<td>1.15 ± 0.19</td>
<td>0.95 ± 0.13</td>
<td>2.33 ± 0.67*</td>
<td>2.03 ± 0.28**</td>
<td>2.13 ± 0.10***</td>
<td>1.97 ± 0.30**</td>
</tr>
</tbody>
</table>
compared to Braak 0; no other significant differential iPS expression was observed (Table 2A-B). *Post mortem* delay and sex did not correlate with any cPS or iPS expression. To analyze whether iPS and cPS expression was affected by age, we correlated amyloid *O* control donors with age and found no significant correlation. To analyze iPS protein expression in relation to Aβ plaques in human tissue, we
performed immunostainings of β1i, β5i, and Aβ (6E10) on post-mortem tissue of hippocampal / entorhinal cortex from control (Braak 0, Amyloid score O) and AD tissue (Braak 5, Amyloid score C). Despite the amyloid score O, some early Aβ plaques were observed in one donor, most of these plaques were surrounded by glial cells with increased immunostaining for β1i (Fig. 3A) and β5i (Fig. 3C), while non-plaque areas showed low β1i and β5i staining. In AD tissue, the expression of β1i (Fig. 3B, E) and β5i (Fig. 3D, F) was increased throughout the tissue, not only around plaques, but also in areas more distant from plaques. The increased staining was especially confined to cells with glial morphology. Double staining with GFAP showed a large overlap with both β1i and β5i, while the microglia marker, HLA-DR showed some overlap although to a lesser degree compared to GFAP (Supplemental Fig. 6 and 7).

**Immunoproteasome activity is increased with increased plaque load in the AD mouse**

Homogenates from cortex of AD and age-matched WT control mice were analyzed using an active-site ELISA [130], allowing an assessment of subunit-specific PS activity for the different cPS and iPS subunits. Three groups of AD animals were analyzed, one with low plaque load (3-6 months); intermediate plaque load (9-12 months), and high plaque load (15-18 months) [6]. All three iPS activities (β5i, β1i, β2i) were significantly elevated in AD mice compared to their age-matched WT groups in the 9-12 month group and the 15-18 month group; β5i (Fig. 4B-C); β1i...
Table 2  Expression fold change of proteolytic proteasome subunits in human AD donors compared to control donors

<table>
<thead>
<tr>
<th>A</th>
<th>Braak 1</th>
<th>Braak 2</th>
<th>Braak 3</th>
<th>Braak 4</th>
<th>Braak 5</th>
<th>Braak 6</th>
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</thead>
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<tr>
<td>Proteolytic constitutive β subunits</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PSMB5 (β5)</td>
<td>0.76 ± 0.03</td>
<td>0.62 ± 0.03*</td>
<td>0.68 ± 0.16</td>
<td>0.69 ± 0.08</td>
<td>0.64 ± 0.04*</td>
<td>0.62 ± 0.03**</td>
</tr>
<tr>
<td>PSMB6 (β1)</td>
<td>0.90 ± 0.05</td>
<td>0.90 ± 0.07</td>
<td>0.92 ± 0.12</td>
<td>1.05 ± 0.09</td>
<td>0.94 ± 0.05</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>PSMB7 (β2)</td>
<td>0.91 ± 0.06</td>
<td>0.75 ± 0.04</td>
<td>0.95 ± 0.12</td>
<td>0.84 ± 0.03</td>
<td>0.90 ± 0.06</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>Proteolytic immunoproteasome β subunits</td>
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</tr>
<tr>
<td>PSMB8 (β5i)</td>
<td>1.21 ± 0.14</td>
<td>0.98 ± 0.10</td>
<td>1.12 ± 0.11</td>
<td>1.29 ± 0.08</td>
<td>1.42 ± 0.08*</td>
<td>1.40 ± 0.26</td>
</tr>
<tr>
<td>PSMB9 (β1i)</td>
<td>1.18 ± 0.16</td>
<td>0.90 ± 0.13</td>
<td>0.87 ± 0.08</td>
<td>1.22 ± 0.20</td>
<td>1.17 ± 0.11</td>
<td>1.13 ± 0.19</td>
</tr>
<tr>
<td>PSMB10 (β2i)</td>
<td>1.24 ± 0.06</td>
<td>1.67 ± 0.20**</td>
<td>1.42 ± 0.14</td>
<td>1.39 ± 0.14</td>
<td>1.17 ± 0.04</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>B</td>
<td>amyloid A</td>
<td>amyloid B</td>
<td>amyloid C</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Proteolytic constitutive β subunits</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>PSMB5 (β5)</td>
<td>0.80 ± 0.07</td>
<td>0.64 ± 0.05**</td>
<td>0.65 ± 0.02***</td>
<td></td>
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</tr>
<tr>
<td>PSMB6 (β1)</td>
<td>0.84 ± 0.11</td>
<td>0.82 ± 0.07</td>
<td>0.84 ± 0.03</td>
<td></td>
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</tr>
<tr>
<td>PSMB7 (β2)</td>
<td>0.92 ± 0.06</td>
<td>0.79 ± 0.06</td>
<td>0.79 ± 0.03</td>
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<tr>
<td>Proteolytic immunoproteasome β subunits</td>
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</tr>
<tr>
<td>PSMB8 (β5i)</td>
<td>1.25 ± 0.18</td>
<td>1.19 ± 0.11</td>
<td>1.40 ± 0.10*</td>
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<tr>
<td>PSMB9 (β1i)</td>
<td>1.30 ± 0.23</td>
<td>1.12 ± 0.13</td>
<td>1.11 ± 0.09</td>
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<tr>
<td>PSMB10 (β2i)</td>
<td>0.93 ± 0.09</td>
<td>1.15 ± 0.08</td>
<td>1.00 ± 0.05</td>
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</tbody>
</table>

*p < 0.05, **p<0.01, ***p<0.001
(Fig. 4E-F) and β2i (Fig. 4H-I). The highest increase was observed in β1i with 272% compared to WT littermates at 15-18 months (Fig. 4F). No change in the β5 and β1 activity of the cPS was observed (Fig. 4A, D), while the β2 activity was elevated in AD mice compared to WT littermates at 9-12 months and at 15-18 months (Fig. 4G, I). No significant decrease of PS activities could be observed in ageing WT or AD mice. Furthermore, an age-dependent, significant increase in β5i, β1i, and β2i activity was observed in WT mice of 15-18 months of age compared to younger 3-6 months WT mice (Fig. 4B, E, H). The β2 activity was found increased in aged WT of 9-12 and 15-18 months compared to younger WT of 3-6 months (Fig. 4G).

Additionally, we assessed the PS activity with the conventional AMC-peptide hydrolysis method used in previous studies reporting decreased PS activity [116, 119–123]. Cleavage of three commonly used proteasome substrates was analyzed to assess C-L, CT-L, and T-L activity in cortical homogenates of AD and WT mice of different ages. The C-L activity was decreased by 27% in AD mice at 15 months (Fig. 4J) and the CT-L activity was decreased by 17% and 18% at 15 and 18 months (Fig. 4K), compared to age-matched WT mice. The T-L did not differ between AD and WT mice (Fig. 4L). Additionally, no impairment of PS activity could be observed when crossing the APPswePS1dE9 mouse with the UbG76VGFP PS activity reporter mouse. (Supplemental Fig. 8A-C). Thus, the decrease in activity as measured by the AMC peptides assays is likely to be a proteasome-independent effect.

**Immunoproteasome activity is increased in human AD samples**

A total of 64 human control and AD post mortem hippocampal tissue samples were selected based on tau and amyloid scores and analyzed for subunit specific cPS

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Table 2. Changes in expression of proteolytic proteasome subunits in human hippocampus of nondemented controls and AD. Table 2 shows mean fold changes (FC; compared to Braak 0, or amyloid O controls) ± SEM of normalized gene expression quantified by RT-qPCR. The different proteolytic proteasome subunits were compared between tissue showing AD pathology (based on either (A) Tau score (Braak stage) [168] or (B) Amyloid score [169] and control tissue where no AD pathology was observed (Braak score 0 or Amyloid score O). Donors per Braak stage; n=9-23, and amyloid score; n= 8-35. Significance was tested using Kruskal-Wallis test with Dunn’s multiple comparison test, * p<0.05, **p<0.01, ***P<0.001.
Figure 4. The immunoproteasome activity is increased with age and plaque load in AD mice. Proteasome activity in cortex of AD and WT mice was measured with an activity ELISA (ProCISE) (A-I) and peptide hydrolysis assays (J-L). Proteasome activity of cPS and iPS subunits was measured in homogenates of APPswePS1dE9 and WT mice of different ages. A, D and G show cPS activities and B, E, and H show iPS activities in RFU/protein concentration. Change in activity is shown as % of age-matched WT in (C, F, and I). No change in cPS β5 activity (A, C) or β1 activity (D, F) was observed between AD and WT mice in any of the age groups. The β2 cPS activity was increased in AD mice compared to WT mice at 9-12 months and at 15-18 months and in aged WT mice compared younger WT mice (G, I). All three iPS activities were increased in AD mice compared to age matched WT mice. The β5i activity (B, C); the β1i activity (E, F) and the β2i activity (H, I) were increased in AD at 9-12 months and at 15-18 months. All three iPS activities were increased in aged WT mice compared to younger WT
and iPS activity, as previously done in the mouse samples. When grouping samples on Braak tau scores, no difference in any of the cPS activities was observed between the different Braak stages (Fig. 5A-C). In contrast, all the three iPS activities were elevated with increased tau pathology; the β1i activity was increased by 82% at Braak 4 compared to Braak 0 controls (Fig. 5D). The β2i activity was increased at Braak 4, 5, and 6 groups compared to the Braak 0 controls with largest increase of 59% at Braak 5; the Braak 6 group also differed significantly from the Braak 1 group (Fig. 5E). The β5i activity was significantly increased in Braak 4, and 6 compared to Braak 0, with the highest increase of 62% at Braak 6 (Fig. 5F).

When grouping the samples based on amyloid scores, the amyloid score A and B did not differ significantly and were pooled due to their low sample numbers. The β2 activity was increased by 15% in amyloid score C compared to amyloid O controls (Fig. 5H), while the β5 activity showed a transient impairment of 14% in the amyloid A+B, but remained unchanged at amyloid C compared to amyloid O (Fig. 5I). All iPS activities (β5i, β1i, β2i) were significantly higher in the amyloid C group compared to O group (44%; 39%; 46%, respectively). The β1i and β5i activities were also increased compared to the amyloid A+B group (Fig. 5J-L). After correcting for age, using a partial correlation analysis, all the iPS activities showed a positive correlation for amyloid score, the β5i and β2i activities significant while the β1i activity showed a near significant correlation (Pearsons’ r; β5i: 0.536 (p<0.0001); β2i: 0.543 (p<0.0001) and β1i: 0.283 (p=0.054); for Braak tau score the β5i and β2i activities showed a positive significant correlation (Pearsons’ r; β5i: 0.554 (p<0.001); β2i: 0.630 (p<0.001)). No significant correlation between the activity of the cPS subunits mice (B, E, H). Figures (J-L) show the PS activity measured in the same homogenates using the peptide hydrolysis method, using activity specific AMC-conjugated probes. (J) Caspase activity showed impaired activity at 15 months. (K) Chymotryptic activity was decreased in 15 and 18 months AD mice. (L) The TL measured did not show any significant changes between WT and AD. In (A, B, D, E, G, H) the Y-axis indicates relative fluorescent units normalized on protein load, black lines mark the group mean, error bars show SEM. In (C, F, I) the y-axis shows activity in % of age matched WT, the bars show the group mean, error bars show SEM, n=5-6. Significance was tested using Mann-Whitney U test. In (J-L), bars show mean of proteolytic activity in % of WT littermate controls, error bars show SEM, n= 8-9 and significance was tested using Students paired t-test on AD and WT littermate couples assayed at the same time. * p<0.05, **p<0.01, ***p<0.001.
Figure 5. The immunoproteasome activity is increased with AD pathology in human post mortem tissue. Proteasome activity of cPS and iPS subunits was assessed in human post-mortem hippocampal/entorhinal cortex tissue from controls and AD donors using the ProCISE assay. (A-C) Show the cPS activities grouped on Braak (Tau) staging. (G-I) show the cPS activities grouped on amyloid score, amyloid score A and B were pooled in this analysis. (J-L) show the different iPS activities grouped on amyloid score; all three subunits showed increased activity in the amyloid C group compared to O. Bars show mean of subunit activity.
and tau or amyloid score was observed.

**Inhibition of β5i activity reduced pro-inflammatory signaling in microglia isolated from AD and WT mice.**

As shown in Fig. 1E, stimulation with LPS increased PS activity in microglia. LPS acts via the CD14 receptor to induce innate immune signaling [170]. CD14 and TLR2 are also Aβ recognition receptors, involved in Aβ induced activation of innate immune signaling [171, 172]. To investigate whether CD14 and TLR2 were implicated in the plaque pathology of this AD mouse model, we analyzed their gene expression in the cortex. Elevated levels of both Cd14 and Tlr2 transcripts were observed in AD compared to WT mice after the emergence of plaques, starting from 9 months and onward (Fig. 6A-B). Previously, inhibition of β5i iPS activity been shown that reduced innate pro-inflammatory signaling in peripheral immune cells [130]. We hypothesized that an inhibition of the elevated β5i activity in AD microglia, would lead to a decrease in pro-inflammatory signaling. Therefore, microglia from 15 months old AD and WT mice were acutely isolated and treated ex-vivo with specific β5 or β5i inhibitors (as used in previous studies [128, 130]). Inhibition was followed by LPS stimulation to further stimulate the CD14-mediated signaling pathways (for scheme see Fig. 6C). Very low or undetectable levels of Cd14, Tnf, and Il1b was observed in non-LPS stimulated AD or WT microglia (Fig. 6D, F, G). Firstly, upon LPS stimulation we observed that microglia from AD mice showed higher mRNA expression of Cd14, Tlr2, and Il1b compared to WT microglia (Fig. 6D, E, G). Secondly, inhibition of the enhanced β5i activity using ONX0914 led to a significant attenuation of Cd14, Tlr2, and Il1b expression in response to LPS stimulation (Fig. 6D, E, H). Thirdly, Tnf mRNA expression was equally elevated after LPS stimulation in both AD and WT microglia. Here, inhibition of β5i activity caused a significant reduction of LPS induced Tnf expression in both groups (Fig. 6F). In most cases, inhibition of the constitutive

figure text cont. (Fig. 5)
Figure 6. Specific inhibition of the iPS β5i activity reduced pro-inflammatory signaling in microglia isolated from aged AD and WT mice. (A-B) The receptors CD14 and TLR2 have been implicated in recognition of Aβ and activation of innate immune signaling. The levels of Cd14 and Tlr2 were elevated in cortex of AD mice compared to WT mice, starting from 9 months, n=6-9, bars show normalized mRNA expression. (C) Schematic overview of inhibition and stimulation scheme of isolated microglia from 15 months old AD and WT mice. β5i and β5 activity was inhibited using specific inhibitors (ONX0914 for β5i, PR-825 for β5), prior LPS stimulation of isolated AD and WT microglia. (D-E) Cd14 and Tlr2 expression were higher in AD microglia compared to WT microglia after LPS stimulation. Inhibition of β5i activity prevented the LPS induced Cd14 and Tlr2 expression in AD microglia to similar levels as of the non-LPS-stimulated microglia. (F) No difference in induction of Tnf expression was observed between AD and WT microglia. Here, β5i inhibition reduced the Tnf levels in both groups. (G) Il1b expression was low in WT microglia, undetectable in a few samples. Microglia isolated from AD mice showed increased expression of Il1b after LPS stimulation; which was strongly reduced in the β5i-inhibited samples (H) compared to vehicle treated samples. In all cases, inhibition of β5 had no significant effect on the expression of these genes. (D-G) Bars show normalized gene expression, error bars show SEM, n=5 (no LPS n=2), significance tested using Mann Whitney U test. (H) Bars show fold over vehicle control, error bars SEM, significance tested using one sample t-test.
β5 activity using PR-825 led to a slight, but non-significant, reduction of gene expression compared to vehicle control (Fig. 6D-F). These data accentuates the strong involvement of the iPS, especially the β5i subunit in regulating the elevated innate immune signaling observed in this AD mouse model.

**Discussion**

The present study for the first time reports in detail on the activity of all PS subunits in an AD mouse model and in human post-mortem brain tissue of non-demented controls and patients with different stages of AD. We reveal a clear increase of iPS activity in both the AD mouse and in human AD tissue using a novel and highly sensitive method to assess subunit-specific proteasome activity. The transcript levels of the iPS subunits were also elevated in mice with a higher plaque-load, but remained mainly unregulated in the human AD tissue, in contrast to their corresponding activity. Moreover, we found no impairment of activity in any of the iPS or cPS subunits in the AD mouse and in human AD pathology, a part from a slight transient impairment of the β5 activity, at amyloid score A+B in the human tissue. Our findings are in contrast to the impairment of PS activity observed in all earlier studies in brain homogenates of human AD and mouse AD brain samples [116, 119–123]. Together with accumulation of ubiquitinated proteins in AD brains, this led to the widely held belief that proteasome inhibition is a prominent feature of AD pathology [173]. Here we show that ubiquitin accumulation is confined to dystrophic neurites around plaques in the AD mouse, and not present in glia. However since we do not observe any reduction in iPS or cPS activity, we argue that the accumulation dystrophic neurites is not due to a decreased PS activity. The previous PS activity studies were all based on hydrolysis of AMC-conjugated peptides; therefore we also used this method here, to assess PS activity, along with the subunit-specific activity-ELISA. Using the AMC peptides, we found a slight decrease in CT-L and C-L PS activity in older AD mice. Though this finding is in line with previous studies, it is in contrast to the results of the activity-ELISA assay. The discrepancy between the two methods is likely to be due to several limitations of the AMC peptide hydrolysis assays. One problem is its relative aspecificity [174], as the AMC substrates can also be cleaved by other
proteases, like calpains and caspases [175]. Furthermore, the AMC-peptides cannot
distinguish between cPS and iPS subunit activity [109, 175], while the activity-ELISA
assay is able to do so.

In contrast to the previously published PS activity data, protein levels of
the iPS were reported to be elevated in AD brains by Western blot and immuno-
histochemistry [116, 117]. Also in other diseases with strong reactive gliosis, such
as multiple sclerosis [176], AIDS dementia [177], and epilepsy [110] increased iPS
levels were found. It has been suggested that the induction of iPS could “hijack” the
function of the cPS, leading to a dysfunction of the latter [177]. However, such an
effect cannot be confirmed here, since the upregulation of iPS activities did not coin-
cide with a significant impairment of the cPS activities in either the AD mice or in the
human AD tissue.

Using the same model as in our study, Aso and colleagues found elevated
protein levels of β1i and β2i, together with a reduction in the CT-L activity in aged AD
mice. This led the authors to argue that the iPS was dysfunctional [108]. An increased
expression of iPS has also been observed in chronically inflamed tissues such as
inflammatory bowel disease [178] and chronic hepatitis [179], the latter concomitant
with sustained TLR activity. Here we showed an increase in iPS activity together with
an increased iPS staining of glia around plaques in AD tissue (Mouse and Human),
in the AD mouse we also demonstrate that this upregulation is confined to plaques
by analyzing microdissected plaque and non-plaque areas. In addition, we show that
cell cultures treated with Aβ show increased proteasome activity, especially primary
microglia. All these observations indicate that Aβ deposition underlies the increased
iPS expression, most likely in response to an Aβ-induced TLR/CD14 receptor activa-
tion [26, 171, 180] as discussed below. To further prove that TLR/CD14 signaling
can bring about increased iPS expression and PS activity, we stimulated microglia
with LPS and observed a strong induction of predominantly, iPS subunits, and an
increased PS activity.

The iPS is known to have a role in pro-inflammatory cytokine regulation
involved in the innate immune response, believed to be mediated by NF-κB
transcription, although the exact mechanism is not completely known [95, 126]. An
increased PS activity and elevated iPS levels has previously been shown upon IFNγ
stimulation [92], and in mouse brains after a cytotoxic T-cell injury, where increased iPS expression in microglia, astrocytes, and oligodendrocytes was observed [109]. Aβ-induced activation of NF-κB has been shown in astrocytes [32] and in microglia [31]. The increase of iPS activity observed, together with elevated pro-inflammatory signaling in AD mice [6, 65, 181], is likely to be a response to an activation of the innate immune response by Aβ. This process might be mediated by CD14, which has been shown to be involved in Aβ recognition in microglia cells [171]. We show that Cd14 expression is elevated in relation to plaque load. Taken together, these data suggest that the reactive glia cells, surrounding plaques may have entered a long-lasting, pro-inflammatory state, with increased iPS expression and activity, likely via sustained TLR2/CD14-dependent signaling. This may have detrimental effects on the homeostatic functioning of astrocytes [182] and is likely to contribute to neuronal excitotoxicity [183]. Introduction of a CD14 null mutation in the APPswePS1dE9 line, led to a reduction of plaque load, implicating that a reduction of CD14 levels is beneficial for plaque clearance [184]. Additionally, a deletion of TLR2 in monocytes and microglia reduced cytokine release and improved Aβ phagocytosis [172]. While AD mice with a global TLR2 deficiency, showed impaired memory and elevated Aβ levels [67]. An initial activation of the innate immune response may be beneficial for Aβ clearance [185], therefore increased iPS levels may be beneficial to enhance the degradation of oxidized and damaged proteins that accumulate under acute inflammatory cell stress [145, 146]. However, a sustained dysregulation of immune signaling is a known feature of AD and is likely to be a key contributor in the pathophysiology of AD (as reviewed in [66]). Sustained immune signaling has previously been shown to lead to reduced levels of Aβ recognition receptors and of Aβ degrading enzymes in microglia from aged AD mice [65], as well as a reduction of general cognitive functions [186]. A continuously elevated iPS activity may be accountable for the sustained innate immune response, which in turn contributes to glial dysfunction and increased oxidative stress (the latter- Orre et al., unpublished observations [chapter 1]). A mild inhibition of the proteasome attenuated tissue damage and had anti-oxidative effects in a rat model of diabetic nephropathy [153]. Specific inhibition of the β5i activity reduces levels of pro-inflammatory molecules and tissue damage in a rheumatoid arthritis mouse model and in a model of inflammatory
bowel disease [128, 130]. Moreover, LPS stimulation of macrophages isolated from LMP7/MECL-1 (β5i/β2i) knockout mice was associated with suppression of cytokine secretion [187]. Here we show that inhibition of the elevated β5i activity, for the first time in brain cells, leads to a reduction of several innate immune signaling molecules and receptors. These results clearly indicate that β5i inhibitors possess anti-inflammatory properties.

This study provides a paradigm shifting view on the function of the UPS in relation to plaque pathology in AD, and points out to its involvement in neuroinflammation and its association with glial reactivity. To target the elevated β5i activity with a β5i specific inhibitor, in vivo, would be a potential strategy to reduce the sustained pro-inflammatory signaling in AD, without affecting other essential functions of the UPS. Additional studies to further investigate this novel interaction between iPS, Aβ induced glial reactivity, and neuroinflammation, are needed to fully elucidate their relationship in AD.

**Supplemental data**

For supplemental data please go to [http://www.ellyhollab.eu/](http://www.ellyhollab.eu/).