



Reduced amyloid- β degradation in early Alzheimer's disease but not in the APPswePS1dE9 and 3xTg-AD mouse models

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Summary

Alzheimer's disease (AD) is hallmarked by amyloid- β (A β) peptides accumulation and aggregation in extracellular plaques, preceded by intracellular accumulation. We examined whether intracellular A β can be cleared by cytosolic peptidases and whether this capacity is affected during progression of sporadic AD (sAD) in humans and in the commonly used APPswePS1dE9 and 3xTg-AD mouse models. A quenched A β peptide that becomes fluorescent upon degradation was used to screen for A β -degrading cytoplasmic peptidases cleaving the aggregation-prone KLVFF region of the peptide. In addition, this quenched peptide was used to analyze A β -degrading capacity in the hippocampus of sAD patients with different Braak stages as well as APPswePS1dE9 and 3xTg-AD mice. Insulin-degrading enzyme (IDE) was found to be the main peptidase that degrades cytoplasmic, monomeric A β . Oligomerization of A β prevents its clearance by IDE. Intriguingly, the A β -degrading capacity decreases already during the earliest Braak stages of sAD, and this decline correlates with IDE protein levels, but not with mRNA levels. This suggests that decreased IDE levels could contribute to early sAD. In contrast to the human data, the commonly used APPswePS1dE9 and 3xTg-AD mouse models do not show altered A β degradation and IDE levels with AD progression, raising doubts whether mouse models that overproduce A β peptides are representative for human sAD.

Key words: APPswePS1dE9; Alzheimer; amyloid; insulin-degrading enzyme; 3xTg-AD.

Introduction

Alzheimer's disease (AD) is hallmarked by extracellular deposits of amyloid- β (A β) peptides in plaques and intracellular neurofibrillary

tangles containing hyperphosphorylated tau. According to the amyloid-cascade hypothesis, A β accumulation in the brain drives further AD pathogenesis, such as tau aggregation, synaptic dysfunction, and eventually neuronal death (Hardy & Allsop, 1991; Murrell *et al.*, 1991; Hardy & Selkoe, 2002; Querfurth & LaFerla, 2010; Tam & Pasternak, 2012). The progression of AD is classified by different Braak stages and amyloid scores (Braak & Braak, 1991), reflecting the distribution of plaques and tangles in well-defined brain areas. A β peptides are generated from the transmembrane amyloid precursor protein (APP) by β -secretase and γ -secretase in organelles including the endoplasmic reticulum, Golgi apparatus, and the endosomal-lysosomal pathway (Selkoe, 2001; LaFerla *et al.*, 2007). Predominantly, A β peptides of 40 (A β ₄₀) or 42 amino acids (A β ₄₂) are generated, with A β ₄₂ being more aggregation prone (Thinakaran & Koo, 2008). While extracellular A β aggregation has long been considered as the primary cause of AD, intracellular A β accumulation is detected in neurons prior to the appearance of extracellular deposits (Wirths *et al.*, 2001; Youmans *et al.*, 2012) and is associated with cytotoxicity, dysfunction of organelles, and neurodegeneration (Bayer & Wirths, 2010). Translocation of A β peptides into the cytoplasm could occur via various routes (Li *et al.*, 2007), including transport of ER-generated A β peptides into the cytoplasm via systems related to ER-associated degradation (ERAD) (Bückig *et al.*, 2002; Schmitz *et al.*, 2004), passive leakage of A β along the secretory pathway, or by membrane permeability of lysosomes containing internalized A β (Yang *et al.*, 1998).

While the rare autosomal dominant familial AD (fAD) is mostly due to overproduction of A β (Bertram *et al.*, 2010; O'Brien & Wong, 2011) or enhancing A β protofibril formation (Nilsberth *et al.*, 2001), far more common is late-onset sporadic AD (sAD), thought to be caused by decreased clearance of the A β peptide (Dorfman *et al.*, 2010; Mawuenyega *et al.*, 2010). Several proteases are able to degrade A β and especially insulin-degrading enzyme (IDE), also named insulysin, is extensively described for its role as an A β -degrading enzyme (Kurochkin & Goto, 1994; Qiu *et al.*, 1998; Pérez *et al.*, 2000; Vekrellis *et al.*, 2000; Caccamo *et al.*, 2005). IDE is mainly cytoplasmic (Kurochkin & Goto, 1994) but also has a neuronal transmembrane isoform (Vekrellis *et al.*, 2000) is found in peroxisomes (Chesneau *et al.*, 1997) and can be secreted via exosomes by microglia (Qiu *et al.*, 1998) and astrocytes (Jiang *et al.*, 2008). Knock down of IDE levels in HeLa cells was shown to result in accumulation of cytoplasmic A β peptides after they were transported to the cytoplasm via ERAD (Schmitz *et al.*, 2004), subsequently capable of inducing cell death (Lee *et al.*, 2006).

Our study aims at elucidating the role for peptidases in cytoplasmic A β clearance during different stages in the development of AD, in both human postmortem brains and brains of the commonly used APPswePS1dE9 and 3xTg-AD mouse models. Using a quenched A β peptide that becomes fluorescent upon cleavage

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inside the KLVFF region critical for A β peptide aggregation, we confirmed IDE to be the main peptidase that degrades cytoplasmic A β . More important, we found that the capacity to degrade A β is already dramatically decreased during the earliest stages of sAD in humans. This decline correlated with IDE protein levels but not RNA levels. Interestingly, these changes in IDE levels and A β degradation were not observed in the APPswePS1dE9 and 3xTg-AD mouse models, raising doubts whether mouse models that overproduce A β peptides mimic the alterations in A β clearance in human sAD.

Results

IDE is the main cytoplasmic peptidase degrading the monomeric A β peptide

We developed a sensitive A β degradation assay by generating a quenched A β_{40} peptide containing a small fluorescein group and a quenching dabcyI group (Reits *et al.*, 2004) flanking the KLVFF sequence present in the middle of the A β peptides that is a critical region for aggregation (Fig. 1A) (Tjernberg *et al.*, 1996). The quenched A β_{40} peptide (qA β_{40}) only becomes fluorescent upon separation of quencher and fluorophore, hence after degradation of A β . The qA β_{40} peptides were added to cytoplasmic fractions of mildly lysed HEK293 cells, thereby preventing contamination with extracellular membrane-bound or lysosomal proteases, resulting in an increase in fluorescent signal (Fig. 1B). To identify which proteases play a role in this A β clearance machinery, different

protease inhibitors were added to the cytosolic fractions prior to adding the qA β_{40} . The proteasome inhibitor MG132 did not show any effect on A β degradation, nor did inhibition of cysteine peptidases (E64), bestatin-sensitive aminopeptidases, serine proteases (PMSF), aspartyl proteases (pepstatin), puromycin-sensitive aminopeptidase (PAQ22), tripeptidyl-peptidase II (butabindide, AAF-CMK), thimet oligopeptidase (Cpp-AAF-pNa), or nardilysin (amasatin). However, inhibiting metalloproteases by adding phenanthroline significantly decreased the rate of qA β_{40} degradation. Specific inhibition of the metallopeptidase IDE using bacitracin reduced qA β_{40} degradation (Fig. 1C) and as no additional decrease was observed upon combining phenanthroline and bacitracin, this indicates that IDE is the main cytoplasmic protease responsible for efficient A β degradation.

As most peptidases can only target monomeric A β , inhibition of IDE, and the resulting decrease in degradation rate of qA β_{40} , could accelerate aggregation of the nondegraded qA β_{40} . Therefore, the effect of A β oligomerization on degradation rates was investigated. The qA β_{40} peptide was preincubated at 37 °C to allow oligomerization for 2–6 h before addition to the cytosolic fraction. Reduced fluorescent levels were found with preincubated qA β_{40} peptides (Fig. 1D), suggesting that aggregation of qA β_{40} peptides prevents its degradation. To exclude that this effect was due to impeding of IDE rather than an inability to degrade oligomeric forms of qA β_{40} , monomeric or 2 h preincubated oligomeric qA β_{40} was added to cytosolic fractions. When no degradation occurred anymore, indicated by reaching the plateau level of fluorescence, a second

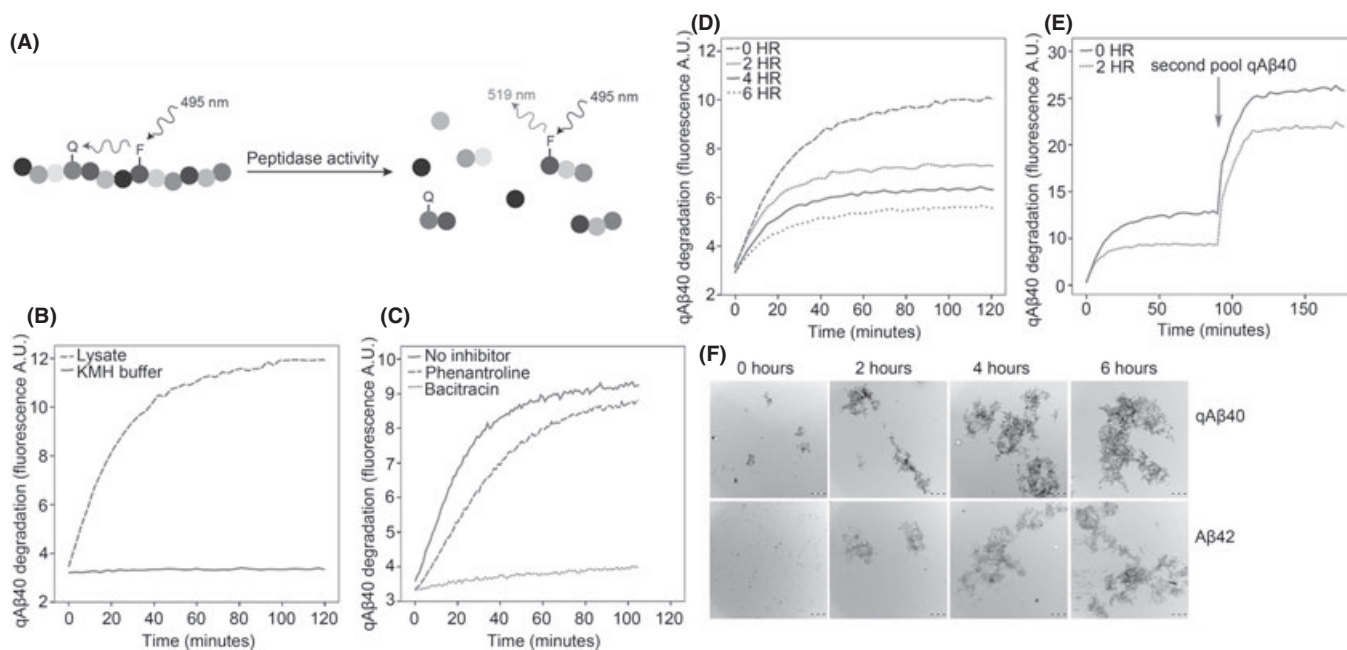


Fig. 1 Monomeric and oligomeric qA β_{40} degradation (A) Fluorescence can only be detected upon degradation of the quenched amyloid- β_{40} (qA β_{40}) peptide when the fluorescein group (F) is separated from the quencher group (Q). (B) qA β_{40} is degraded in HEK293 cytosolic fractions (dashed line) but not in KMH buffer (solid line). (C) qA β_{40} degradation in HEK293 cytosolic fractions without inhibitor (solid line), preincubated with 100 μ M phenanthroline (dashed line) and preincubated with 200 μ M bacitracin (dotted line). (D) Degradation of qA β_{40} that was preincubated at 37 °C for 0 h (dashed line), 2 h (upper dotted grey line), 4 h (solid black line), or 6 h (lower dotted line) in HEK293 cell lysates. (E) Oligomeric A β does not impair insulin-degrading enzyme, as monomeric qA β_{40} is as efficiently degraded in HEK293 cell lysates preincubated with monomeric qA β_{40} (solid line) and with 2 h preincubated qA β_{40} (dotted line). The additional pool of monomeric qA β_{40} was added when the fluorescence plateau level was reached (arrow). (F) Electron microscopy images showing increased oligomerization with time of qA β_{40} and synthetic A β_{42} at 37 °C.

Table 1 Detailed donor information

	Amyloid score	Age	Sex	PMD	pH	BW
Br 0 (<i>n</i> = 9)	6O-2A-1B	60.1 \pm 6.0	2F-7M	7.2 \pm 3.1	5.9 \pm 2.2	1282 \pm 177
Br I (<i>n</i> = 10)	4O-3A-1B-1C-1NA	81.8 \pm 9.4	5F-5M	6.7 \pm 1.9	6.6 \pm 0.3	1239 \pm 144
Br II (<i>n</i> = 9)	3O-2B-2C-2NA	84.8 \pm 6.8	6F-3M	6.0 \pm 0.9	6.5 \pm 0.2	1213 \pm 99
Br III (<i>n</i> = 10)	1O-1A-2B-2C-4NA	85.2 \pm 6.6	6F-4M	5.2 \pm 2.1	6.5 \pm 0.3	1235 \pm 151
Br IV (<i>n</i> = 10)	1B-7C-2NA	85.2 \pm 5.6	5F-5M	5.5 \pm 1.8	6.6 \pm 0.2	1176 \pm 163
Br V (<i>n</i> = 10)	2B-7C-1NA	81.2 \pm 9.4	5F-5M	5.8 \pm 1.4	6.4 \pm 0.2	1138 \pm 95
Br VI (<i>n</i> = 10)	1B-6C-3NA	77.5 \pm 11.4	5F-5M	5.6 \pm 1.7	6.5 \pm 0.2	1072 \pm 143

BW, brain weight (grams); NA, not available; PMD, Postmortem delay (hours).

pool of only monomeric qA β ₄₀ peptides was added to the same lysates. These peptides were equally efficiently degraded (Fig. 1E), demonstrating that IDE did not become impeded by oligomeric qA β ₄₀. Aggregation of qA β ₄₀ was confirmed by electron microscopy, showing increased oligomerization in time, comparable with unmodified A β ₄₂ (Fig. 1F).

A β peptides are less efficiently degraded by IDE with sAD development

As the hippocampus is highly susceptible to A β accumulation, we examined whether the ability to degrade A β is affected in sporadic (sAD) using human hippocampal tissue of postmortem brains with varying Braak stages. Included were Braak 0 (control, no AD) and Braak I–VI. Information regarding postmortem samples is listed in Table 1. When the ability to degrade qA β ₄₀ was determined for each Braak stage (Fig. 2A, solid bars), a strong decrease in qA β ₄₀ degradation rate was already observed in Braak stage I (two-tailed *t*-test, *P* = 0.03), which further decreased during the progression to Braak stage II (two-tailed *t*-test, *P* = 0.01). Intriguingly, in Braak stage III, the ability to degrade qA β ₄₀ was partially restored, only to decrease again in Braak stage IV (two-tailed *t*-test, *P* = 0.02) and further decrease as a function of progression of Braak stage (Braak V: two-tailed *t*-test, *P* = 0.0005, Braak VI: two-tailed *t*-test, *P* = 0.000). In Braak VI, qA β ₄₀ degradation rate decreased with more than 65% compared with Braak 0. When bacitracin was added to hippocampal lysates prior to addition of the qA β ₄₀, the ability to degrade qA β ₄₀ was strongly reduced in all Braak stages upon IDE inhibition (Fig. 2A, dotted bars). This indicates that IDE is also the main protease responsible for A β clearance in human hippocampal lysates and suggests that IDE is impaired in sAD. Also, oligomerized A β was less well degraded with increasing Braak stage (Fig. 2B). AD progression can also be classified by plaque distribution in well-defined brain areas giving a particular amyloid score, ranging from O (no plaques) to C. Similar to increasing Braak stages, the rate of qA β ₄₀ degradation decreased with increasing amyloid score, being significant for hippocampal fractions of brains classified with amyloid score C (two-tailed *t*-test, *P* = 0.017) (Fig. 2C). Whereas qA β ₄₀ degradation rate is significantly negatively correlated with amyloid score and Braak stages (respectively; Pearson's correlation coefficient *r* = −0.499, *P* = 0.000 and *r* = −0.469, *P* = 0.000), qA β ₄₀ degradation rate was not significantly correlated

with postmortem delay, sex, age, and ApoE genotype. When combining Braak stages and amyloid scores, the correlation with qA β ₄₀ degradation was even stronger (Pearson's correlation coefficient *r* = −0.583, *P* = 0.000) (Table 2).

To examine the reduced qA β ₄₀ degradation in sAD in more detail, we examined IDE protein and mRNA levels, because IDE was found to be the main peptidase degrading the qA β ₄₀. Strikingly, a strong decrease in IDE protein levels was observed already in Braak stage I, which further decreased during the progression to Braak stage II, in parallel with the qA β ₄₀ degradation rate. Similarly, IDE protein levels partially restored in Braak stage III, and decreased again as a function of progression of Braak stage from Braak stage IV onwards (Fig. 2D), indicating that decreased IDE protein levels were directly linked to impaired qA β ₄₀ degradation and development of sAD. In contrast, IDE mRNA did not correlate with IDE proteins levels and qA β ₄₀ degradation; mRNA levels did not change with Braak stage (Fig. S2A) and were even upregulated with amyloid score (Fig. S2B), being significant for amyloid score B (two-tailed *t*-test, *P* = 0.03).

A β peptides are efficiently degraded in all stages of AD in the APPswePS1dE9 mice

Several genetically modified mouse models have been generated that mimic human AD pathology, including the APPswePS1dE9 double transgenic mice which coexpress APP695 with the Swedish mutation (K594M/N595L) and the human exon-9-deleted variant of PS1 (PS1-dE9). The APPswe mutation is a favorable substrate for β -secretase, whereas the PS1dE9 mutation alters γ -secretase cleavage, thereby promoting overproduction of A β ₄₂. Consequentially, APPswePS1dE9 mice show increased A β ₄₂ production accompanied by plaque pathology in the brain, becoming evident at the age of 6 months (Jankowsky *et al.*, 2004). APPswePS1dE9 mice are commonly used in AD research for behavioral tests and studying the molecular mechanisms in plaque progression (Oksman *et al.*, 2006; Song *et al.*, 2008; Yan *et al.*, 2009). To examine whether the APPswePS1dE9 mice show a similar decrease in A β degradation rate with progression of AD, qA β ₄₀ degradation was measured using hippocampal lysates of APPswePS1dE9 mice at the age of 3 months (no plaque pathology), 9 months (starting plaque pathology), and 18 months (severe plaque pathology) (Kamphuis *et al.*, 2012b) in parallel with wild-type age-matched controls (*n* = 3 per age group). Strikingly, hippocampal lysates of the APPswePS1dE9 mice at all

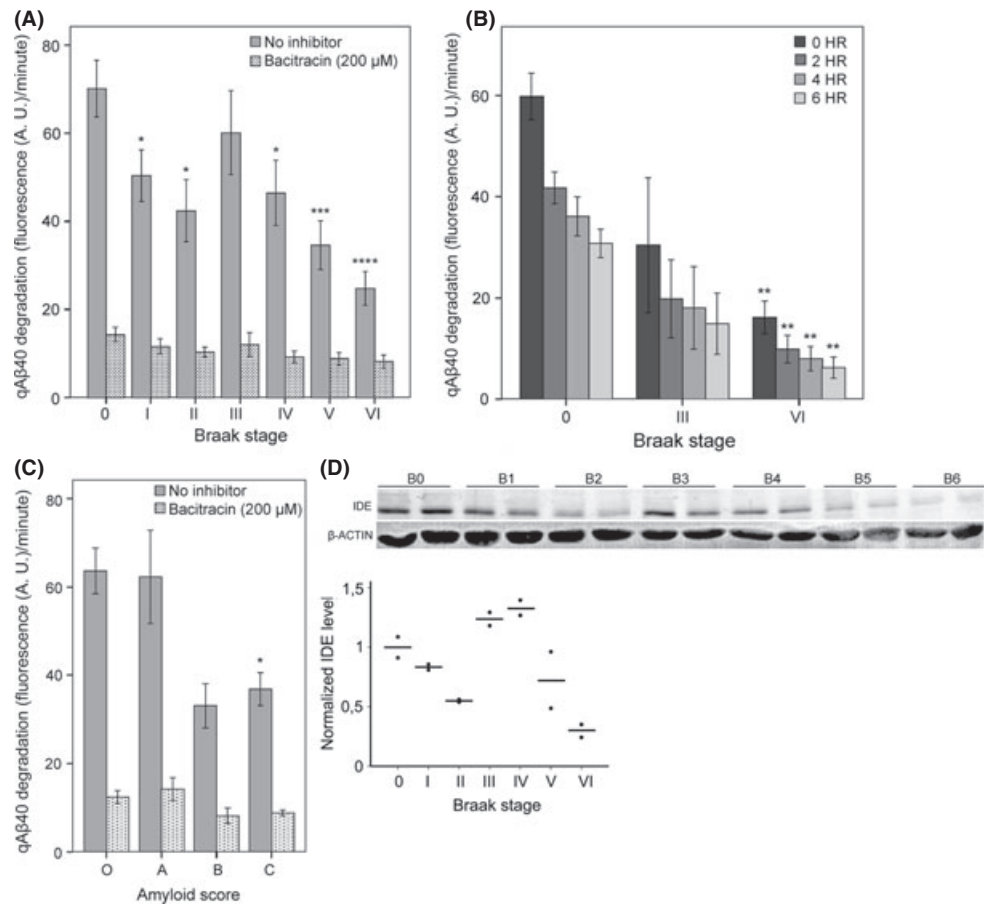


Fig. 2 qA β ₄₀ degradation during the progression of sporadic AD (sAD). (A) qA β ₄₀ degradation in human hippocampal lysates at different Braak stages, without inhibitor (solid bars), and with bacitracin (dotted bars). qA β ₄₀ degradation is significantly decreased in Braak stage I, II, IV, V, and VI. Error bars represent standard error of the mean (SEM) (* P < 0.05; *** P < 0.001; **** P < 0.0001). (B) Degradation of differently preincubated, oligomeric qA β ₄₀ in human hippocampal lysates at Braak stage 0, III, and VI. qA β ₄₀ degradation is significantly decreased in Braak stage VI, at 0 h (P < 0.01), 2 h (P < 0.01), 4 h (P < 0.01), and 6 h (P < 0.01) of preincubation. Error bars represent SEM (** P < 0.01). (C) qA β ₄₀ degradation in human hippocampal lysates at different amyloid scores. qA β ₄₀ degradation is significantly decreased in amyloid score C (P < 0.05). Error bars represent SEM. (D) Upper panel: Western blot analysis of human hippocampal lysates at different Braak stages, showing a decrease of insulin-degrading enzyme (IDE) with increasing Braak stage in sAD. Lower panel: Western blot quantification of IDE protein levels in human hippocampal lysates at different Braak stages.

Table 2 Correlation patient variables and qA β ₄₀ degradation

	Pearson's correlation	P -value
Braak stage	-0.469	0.000
Amyloid score	-0.499	0.000
Braak stage \times Amyloid score	-0.583	0.000
Postmortem delay (h)	0.009	0.941
Age at death	-0.153	0.213
Sex	-0.133	0.281
ApoE	-0.151	0.243

Pearson's correlation values and significance for correlation between qA β ₄₀ degradation and Braak stage, Amyloid score, Braak stage \times Amyloid score, postmortem delay, age at death, sex, and ApoE.

stages of AD showed equal qA β ₄₀ degradation rates as those of age-matched wild-type controls (Fig. 3A), which is in clear contrast with human sAD. As qA β ₄₀ degradation was equally decreased in all lysates upon inhibition of IDE (Fig. 3A), this suggests that IDE is

also the main peptidase responsible for A β degradation in APPswePS1dE9 mice. Just as for the human hippocampal lysates, we measured IDE protein levels in the hippocampal lysates of the APPswePS1dE9 mice at all stages of AD. However, in clear contrast with the human data, neither IDE protein (Fig. 3B) nor mRNA levels (data not shown) change during AD progression in mice. Unaltered IDE protein levels could explain why APPswePS1dE9 mice do not show a decrease in A β -degrading abilities with AD development and indicates that in APPswePS1dE9 mice plaque formation is mainly the result of an increase in A β production.

A β peptides are efficiently degraded in all stages of AD in the 3xTg-AD mice

The triple transgenic mouse model (3xTg-AD) is the first mouse model to develop both plaque and tangle pathology in AD-relevant brain regions (Oddo et al., 2003b) and is often used to study human AD pathology. The 3xTg-AD mice harbor three mutant genes; APP695 with the Swedish mutation (K670M/N671L), PS1_{M146V} and

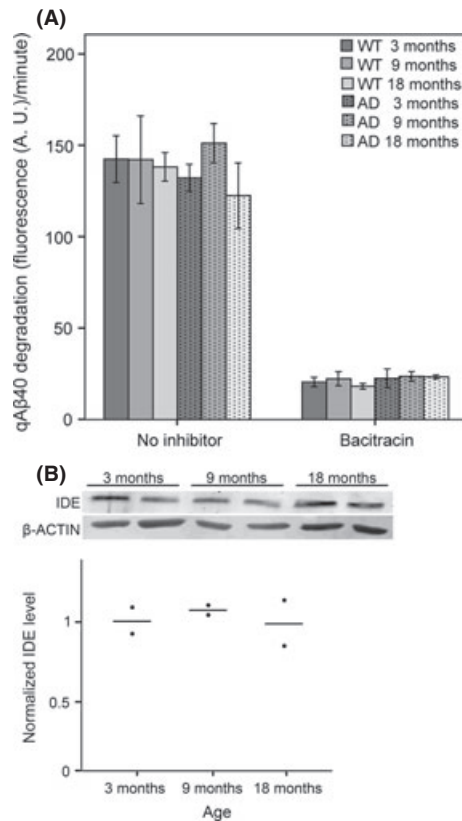


Fig. 3 qA β ₄₀ degradation during the progression of Alzheimer's disease (AD) in the APPswePS1dE9 mice. (A) qA β ₄₀ degradation in mouse hippocampal lysates of both wild-type (solid bars) and APPswePS1dE9 mice (dotted bars) at 3, 9, and 18 months of age. Error bars represent standard error of the mean (SEM). (B) Upper panel: Western blot analysis of APPswePS1dE9 mice hippocampal lysates at 3, 9, and 18 months of age, stained for insulin-degrading enzyme (IDE) and β -actin (loading control). Lower panel: Western blot quantification of IDE protein levels in APPswePS1dE9 mice hippocampal lysates at different ages.

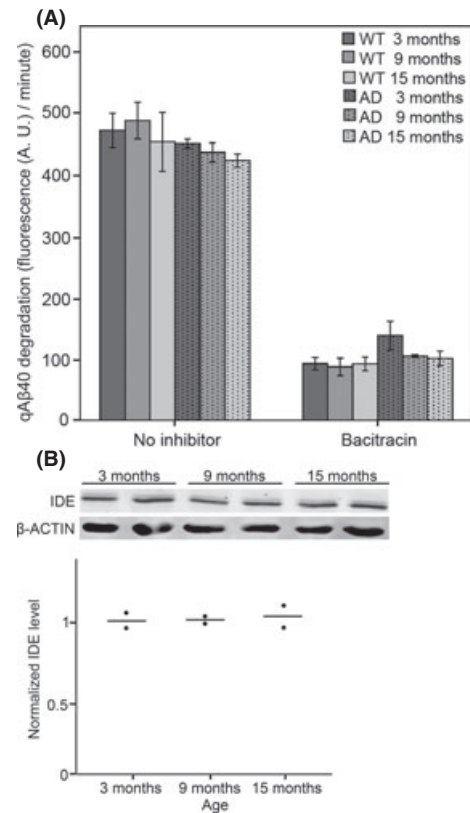


Fig. 4 qA β ₄₀ degradation during the progression of Alzheimer's disease (AD) in the 3xTg-AD mice. (A) qA β ₄₀ degradation in mouse hippocampal lysates of both wild-type (solid bars) and 3xTg-AD mice (dotted bars) at 3, 9, and 15 months of age. Error bars represent standard error of the mean (SEM). (B) Upper panel: Western blot analysis of 3xTg-AD mice hippocampal lysates at 3, 9, and 15 months of age, stained for insulin-degrading enzyme (IDE) and β -actin (loading control). Lower panel: Western blot quantification of IDE protein levels in 3xTg-AD mice hippocampal lysates at different ages.

tau_{P301L}, together promoting the overproduction of A β ₄₂ and tau. Despite equivalent overexpression of human APP and tau, intraneuronal A β accumulation and A β deposition precede tangle pathology, and both pathologies develop in an age-dependent manner with a temporal- and regional-specific profile that closely mimics their development in human AD brain (Oddo *et al.*, 2003a). To examine whether the 3xTg-AD mice mimic the decreased A β degradation rate with progression of AD as observed in the hippocampus of human sAD, we measured qA β ₄₀ degradation rate in hippocampal lysates of 3xTg-AD mice at the age of 3 months (no AD pathology), 9 months (starting AD pathology), 15 months (severe AD pathology), in parallel with wild-type age-matched controls ($n = 3$ per group). Interestingly, just like we observed in the APPswePS1dE9 mouse model, and in clear contrast with the human data, also the 3xTg-AD mice do not show any change in A β degradation capabilities with AD progression; at all stages of AD, the 3xTg-AD mice show equal qA β ₄₀ degradation rates as those of age-matched wild-type controls (Fig. 4A). Inhibition of IDE resulted in decreased qA β ₄₀ degradation, again showing IDE as the main peptidase-degrading A β . As expected from these results, IDE protein levels were equal at all stages of AD (Fig. 4B).

Discussion

In the present study, a unique qA β ₄₀ peptide was used to screen for A β -degrading cytoplasmic peptidases. In addition, this quenched peptide was used to analyze A β degrading capacity in the hippocampus of sAD patients with different Braak stages as well as APPswePS1dE9 and 3xTg-AD mice. Since specifically the KLVFF region in the A β peptide was shown to mediate binding during polymerization and to be essential and sufficient for aggregation (Tjernberg *et al.*, 1996), the quencher and fluorophore were positioned such that they are flanking this important region. A similar approach to generate a quenched A β ₄₂ was unsuccessful due to its high aggregation-prone properties, making it impossible to efficiently solubilize and fluorescently label the synthesized qA β ₄₂ peptides. Using the qA β ₄₀ peptide, A β degrading peptidases could be identified that are specifically cleaving inside the KLVFF region, which is of interest when studying changes in A β degradation underlying the development of AD and consequent plaque pathology. The present study shows IDE as the main peptidase that degrades cytoplasmic monomeric A β , cleaving inside the critical, aggregation-prone KLVFF sequence

as previously suggested by HPLC and mass spectrometry analysis of A β fragments generated by IDE (Chesneau *et al.*, 2000; Guo *et al.*, 2010). Also, neprilysin is described to be able to cleave inside the KLVFF region; however, in the present study, cytoplasmic peptidases were examined, excluding the plasma membrane glycoprotein neprilysin. In addition, we provide evidence that IDE less efficiently degrades oligomeric A β and deficiencies in IDE activity might therefore lead to less A β degradation and subsequent A β oligomerization and accumulation. The present study is the first showing that the capacity to degrade A β is already decreased dramatically in Braak stage I and II of sAD, in contrast to nondemented controls that are efficiently degrading A β . Interestingly, degradation of A β is partly restored in stage III and then again decreased until stage VI. This decrease in A β degradation is in parallel with IDE protein levels, including the temporal revival in stage III. It is tempting to speculate that IDE levels are increased as a rescue mechanism to cope with AD pathology, but might also result from changes in IDE secretion. This pattern has not been described before, probably because previous studies were grouping different-staged AD patients together (Pérez *et al.*, 2000; Zhao *et al.*, 2007; Wang *et al.*, 2010) and because peptidase activities were never investigated for the separate Braak stages. However, our study includes a relatively high number of individuals covering all stages of AD. It seems that the decrease in IDE activity is not the result of a general decrease in peptidase activities, because other peptidases did not show the same pattern of decreasing activity across the Braak stages (data not shown) (Reits *et al.*, 2004). Also, it is unlikely that the observed pattern was caused by competition of the qA β_{40} with increased endogenous A β for degradation by IDE, as endogenous A β levels were a 10 000-fold lower than the added qA β_{40} levels (Roher *et al.*, 2009). In addition, increased endogenous A β levels are found in both hippocampal tissue of AD patients (Matsui *et al.*, 2007), as well as in the APPswePS1dE9 and 3xTg-AD mouse models (Oddo *et al.*, 2003a; Kamphuis *et al.*, 2012b). Together, these observations exclude a significant effect of competition or accelerated aggregation of endogenous A β levels as an alternative explanation for the correlation of IDE levels and A β degradation in sAD.

As intracellular A β accumulation is already occurring prior to plaque formation (Wirths *et al.*, 2001; Youmans *et al.*, 2012) and is described to be associated with cytotoxicity and neurodegeneration (Bayer & Wirths, 2010), this study was mainly interested in identifying cytoplasmic peptidases that are responsible for degrading intracellular A β and may cause A β accumulation when their activity is decreasing. Cytoplasmic IDE was shown to be such a peptidase degrading intracellular A β . However, in addition to its cytoplasmic form, IDE also has a neuronal transmembrane isoform, which is suggested to face the extracellular environment (Vekrellis *et al.*, 2000), is present in peroxisomes (Chesneau *et al.*, 1997), and can be secreted in exosomes by microglia (Qiu *et al.*, 1998) and astrocytes (Jiang *et al.*, 2008). In future studies, it would be interesting to investigate the role of these IDE isoforms and other peptidases present in specific organelles in degrading A β and whether these enzymes show altered levels during the progression to AD.

In addition, the mechanisms underlying the partial recovery of IDE protein levels and A β degradation at Braak stage III remain to be established. It should be noted that stage III is also the stage at which large transcriptional changes occur in human AD brain (Bossers *et al.*, 2010). IDE mRNA levels were not changed with increasing Braak stage and thus did not correspond to altered protein levels of IDE. This suggests that changes in IDE protein levels that were observed in AD brain are caused by changes in IDE half-life or secretion. Interestingly, a similar decrease and temporal increase were observed when measuring qA β_{40} degradation in the cerebrospinal fluid of sAD patients at different stages (manuscript in preparation), indicating that the ability to degrade A β may be globally affected in the central nervous system. Because changes in A β degradation can already be monitored during the earliest stages of the disease, this would provide possibilities to diagnose AD before the appearance of symptoms and the development of plaque pathology.

The present study also provides evidence that the commonly used mouse models APPswePS1dE9 and the 3xTg-AD mice, both showing increased A β_{42} production accompanied by dense-core plaque pathology in the brain (Jankowsky *et al.*, 2004), do not mimic the alterations in A β clearance observed in human sAD. Neither changes in A β degradation with AD development nor changes in IDE levels were observed in these mice. A possible explanation for the observed difference between mice and men could be differences in neuronal loss, as APPswePS1dE9 mice do not show significant neuronal loss (Kamphuis *et al.*, 2012a), although this remains a point of debate (Wirths & Bayer, 2010). The level of neuronal loss in the 3xTg-AD mouse models is also not yet established (LaFerla & Oddo, 2005). In contrast, significant neurodegeneration is thought to occur in the human brain; however, the extent to which neuronal loss occurs in the human hippocampal area in sAD is contradictory (Rossler *et al.*, 2004). Because decreased IDE levels are already observed in Braak stage I and II in which there is not yet any significant neuronal cell loss, a more likely explanation would be the genetic background of the transgenic mice. Here, overproduction of the A β peptide is the primary cause for AD, in contrast to human sAD where decreased clearance of A β seems to be the primary mechanism underlying the development of the disease, also confirming previous data (Dorfman *et al.*, 2010; Mawuenyega *et al.*, 2010). Also, the present study is supporting this view by showing altered levels of IDE in human hippocampal tissue, but not in APPswePS1dE9 and 3xTg-AD mouse hippocampi. Furthermore, these data show that aging per se does not affect the IDE activity and IDE levels, because aging wild-type mice did also not show decreased A β degradation. The comparison of our results from mice and human show that mice data should be considered with caution, as they seem to be not representative for human sAD.

Materials and methods

Human subjects

Human postmortem tissue was obtained from the Netherlands Brain Bank (NBB; Amsterdam, the Netherlands). The brain donors

have given informed consent for using the tissue and for accessing the extensive neuropathological and clinical information for scientific research, in compliance with ethical and legal guidelines (Huitinga *et al.*, 2008). Included in this study were Braak stage 0 ($n = 9$) and I ($n = 10$), II ($n = 9$), III ($n = 10$), IV ($n = 10$), V ($n = 10$), and VI ($n = 10$). Samples were matched as closely as possible for age, sex, postmortem interval, pH-CSF and ApoE genotype. More detailed donor information is presented in Table 1.

Animals

Double APPswePS1dE9 transgenic mice expressed chimeric mouse/human APP containing the K595N/M596L Swedish mutation and human PS1 variant carrying the exon 9 deletion both driven by mouse prion promoter elements, directing the expression to neurons (Jankowsky *et al.*, 2004). For details, see The Jackson Laboratory [strain B6C3-Tg(APPswe, PSEN1-dE9)85Dbo/J; stock number 004462; <http://jaxmice.jax.org/>]. AD mice were maintained as hemizygous and crossed with wild-type C57BL/6. Genotyping was performed by real-time PCR assays specific for the two transgenes and the prion promoter. The 3xTg-AD line was originally generated by comicroinjection of human APP (K670M/N671L) and tau (P301L) transgenes under the control of the Thy 1.2 promoter into mutant PS-1 (M146V) knock-in mice (Oddo *et al.*, 2003b). For details, see The Jackson Laboratory [strain B6;129-Psen1^{tm1Mpm}Tg (APPswe, tauP301L)1Lfa/Mmjax; stock number 004807; <http://jaxmice.jax.org/>]. Wild-type littermates served as controls for the AD animals. All animals were housed under standard conditions with access to water and food *ad libitum*. Animal handling and experimental procedures were reviewed and approved by the ethical committee for animal care and use of experimental animals of the Royal Netherlands Academy for Arts and Sciences, acting in accordance with the European Community Council directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize suffering and number of animals used for the study presented here. Nine APPswePS1dE9 mice and nine wild-type mice were used, each divided in age groups 3 months ($n = 3$), 6 months ($n = 3$), and 18 months ($n = 3$). Also, nine 3xTg-AD mice and again nine wild-type mice were used, each divided in age groups 3 months ($n = 3$), 6 months ($n = 3$), and 15 months ($n = 3$).

Protein isolation

HEK cells were lysed in 25 μ M digitonin (Sigma-Aldrich, St. Louis, MO, USA) in KMH buffer (110 mM KAc, 2 mM MgAc and 20 mM Hepes-KOH, pH 7.2). Human and mouse brain hippocampal tissue was lysed in \pm 400 μ L (adjusted to the amount of tissue) of homogenization buffer [50 mM Tris/HCl pH 7.5, 250 mM sucrose, 5 mM MgCl₂ and freshly added 2 mM ATP, 1 mM DTT, and 0.025% digitonin (Sigma)]. Tissue was homogenized on ice in homogenization buffer using a homogenizer. Cell lysates were incubated for 30 min on ice and centrifuged for 15 min at 20 800 *g* at 4 °C.

Quenched A β peptide degradation assays

Peptide synthesis

An A β ₄₀ peptide DAEFRHDSGYE(**q**)HHQKLFFFA(**f**)DVGSNKGAIILGLMVGGVV containing a fluorophore and quencher was synthesized as described before (Reits *et al.*, 2004), introducing a fluorescein (f) at the cysteine (position 22) and a quenching dabcy group (q) at position 12.

Quenched A β peptide degradation assay

HFIP-treated aliquots of A β ₄₀ were resuspended in DMSO followed by sonication for 10 min, immediately before use. 75 ng (340 nM) of quenched A β ₄₀ was added to 5 μ g protein from the cell lysates in KMH buffer (110 mM KAc, 2 mM MgAc, and 20 mM Hepes-KOH, pH 7.2) to a total volume of 50 μ L. Protease inhibitors [100 μ M 1,10-phenanthroline, 200 μ M bacitracin, 100 μ M E64, 20 μ M MG-132, 100 μ M puromycin, 100 μ M PMSF, 200 μ M pepstatin A (all Sigma), 100 μ M AAF-CMK, 100 μ M amastatin or 50 μ M bestatin (Enzo life sciences AG, Lausen, Switzerland), 50 μ M PAQ-22 (Wako Chemicals, Osaka, Japan), 100 μ M Cpp-AAF-pNa (InstruChem, Manila, Philippines), 50 μ M PAQ-22 (SopaChem, Nieuwegein, The Netherlands), and 100 μ M butabindide (Tocris Bioscience, Ellisville, MO, USA)] were added to the cell lysates and incubated for 30 min at 4 °C. Degradation of the peptide was analyzed at 37 °C using the FLUOstar OPTIMA (BMG Labtec., Jena, Germany).

Electron microscopy

A β peptide preparations were adsorbed on 300-mesh formvar/copper grids for 2 min, and excess fluid was filtered off. Upon staining with 2.5% uranyl acetate for 2 min, grids were analyzed with a Fei technai-12 G2 transmission electron microscope.

RNA isolation and quantitative PCR

RNA from human and mouse hippocampal tissue was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and an overnight precipitation in isopropanol. Total RNA (1.0 μ g) was DNase I treated and used to generate cDNA (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany) using oligo-dT and random hexamer primers. The reverse transcriptase reaction was incubated at 42 °C for 30 min. The resulting cDNA was diluted 1:20 and served as a template in real-time qPCR assays. Real-time qPCR (SYBR® Green PCR Master Mix; Applied Biosystems, Nieuwerkerk, The Netherlands) for IDE was performed using forward primer GGACAGGTTTGCGCAGTTTT and reverse primer ACAGCGTTCACCTCTGTCTTT. Expression levels of human AD samples were normalized against a selection of 10 reference genes (GAPDH, ACTB, PPIA, UBE2D2, EEF1A, RPS27A, AARS, XPNPEP1, RPLP0, and IPO8) based on a geNorm analysis. The normalization factor was the geomean 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. Expression levels of mouse AD samples were normalized against a selection of four reference genes (GAPDH, HPRT, ACTB, and 18S rRNA). The geomean of the reference gene levels was used to normalize the assessed transcript levels of IDE.

Western blotting

Equal protein amounts obtained from the hippocampal cell lysates were separated on 7.5% SDS-PAGE gels. After electrophoresis, proteins were transferred either onto a 0.45- μ m pore size PVDF membrane filter (Schleicher & Schuell, Dassel, Germany). Blots were blocked in 5% dry milk in TBS and incubated with the primary antibodies against IDE (1:500; Abcam, Cambridge, UK) and β -actin (1:500; Abcam) and subsequently with secondary antibodies IRDye 680 or IRDye 800 (1:15,000; LI-COR Biosciences, Lincoln, NE, USA). Signal was detected using the Odyssey imaging system (LI-COR Biosciences).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Peptidases degrading qA β ₄₀.

Fig. S2 IDE mRNA levels with sAD progression.