Prion-like behaviour and tau-dependent cytotoxicity of pyroglutamylated amyloid-β

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Extracellular plaques of amyloid-β and intraneuronal neurofibrillary tangles made from tau are the histopathological signatures of Alzheimer’s disease. Plaques comprise amyloid-β fibrils that assemble from monomeric and oligomeric intermediates, and are prognostic indicators of Alzheimer’s disease. Despite the importance of plaques to Alzheimer’s disease, oligomers are considered to be the principal toxic forms of amyloid-β. Interestingly, many adverse responses to amyloid-β, such as cytotoxicity, microtubule loss, impaired memory and learning, and neuritic degeneration, are greatly amplified by tau expression. Amino-terminally truncated, pyroglutamylated (pE) forms of amyloid-β are strongly associated with Alzheimer’s disease, are more toxic than amyloid-β, residues 1–42 (Aβ1–42) and Aβ1–40, and have been proposed as initiators of Alzheimer’s disease pathogenesis. Here we report a mechanism by which pE-AP may trigger Alzheimer’s disease. Aβ3(pE)–42 co-oligomerizes with excess Aβ1–42 to form metastable low-n oligomers (LNOs) that are structurally distinct and far more cytotoxic to cultured neurons than comparable LNOs made from Aβ3(pE) alone. Tau is required for cytotoxicity, and LNOs comprising 5% Aβ3(pE)–42 plus 95% Aβ1–42 (5% pE-AP) seed new cytotoxic LNOs through multiple serial dilutions into Aβ1–42 monomers in the absence of additional Aβ3(pE)–42. LNOs isolated from human Alzheimer’s disease brain contained Aβ3(pE)–42 and enhanced Aβ3(pE)–42 formation in mice triggered neuron loss and gliosis at 3 months, but not in a tau-null background. We conclude that Aβ3(pE)–42 confers tau-dependent neuronal death and causes template-induced misfolding of Aβ3(pE)–42 into structurally distinct LNOs that propagate by a prion-like mechanism. Our results raise the possibility that Aβ3(pE)–42 acts similarly at a primary step in Alzheimer’s disease pathogenesis.

pE-AP peptides contain an amino-terminal pyroglutamate, whose modification from glutamate is catalysed by glutaminyl cyclase (QC; also known as QPCT). The most prominent pE-AP species in vivo are Aβ3(pE)–40, Aβ3(pE)–42, Aβ1(pE)–40, and Aβ1(pE)–42 (ref. 5; Supplementary Fig. 1). With Aβ3(pE)–42 being most abundant, pE-AP is more cytotoxic and aggregates more rapidly than conventional amyloid-β, and QC activity and pE-AP levels are increased several-fold in Alzheimer’s disease brain. Alzheimer’s disease mouse models also indicate a role for pE-AP in initiating pathology: oral administration of a QC inhibitor led to improved memory and learning, and reduced levels of pE-AP and conventional amyloid-β. These data imply that pE-AP potentiates the neurotoxicity of conventional amyloid-β, but leave open the issue of molecular mechanisms. To address that issue, we compared oligomerization of Aβ3(pE)–42, Aβ1–42, and mixtures of the peptides in vitro, and analysed responses of primary cultured neurons and glial cells (Supplementary Fig. 2) to the oligomers. At 5 μM peptide, 5% pE-AP aggregated faster than Aβ3(pE)–42 or Aβ1–42 alone, but on thioflavin T fluorescence shifts. 31 MAY 2012 | VOL 485 | NATURE | 651

Fig 1. Tau-dependent cytotoxicity of oligomers formed by co-incubation of Aβ3(pE)–42 and Aβ1–42. Primary mouse wild-type (WT) and tau-knockout (KO) forebrain neurons, and secondary cultures of wild-type mouse glia were treated for 12 h with Aβ1–42, Aβ3(pE)–42, or 5% Aβ3(pE)–42 plus 95% Aβ1–42, which were oligomerized for 24 h at 5 μM before dilution into culture media. Cells were exposed to calcine-AM and imaged live by epi-fluorescence microscopy to assay viability. Extensive death and detachment of cells were observed only for wild-type neurons treated with Aβ3(pE)–42, or the 5% Aβ3(pE)–42 plus 95% Aβ1–42, b. Following peptide treatment, cell viability was analysed by the XTT plate reader assay. The robust cytotoxicity of Aβ3(pE)–42 containing solutions at concentrations as low as 0.5 μM, unless Aβ3(pE)–42 and Aβ1–42 were incubated separately during oligomerization (P < 0.01; yellow stars signify statistical significance of the indicated bar graphs versus vehicle controls; black stars signify statistical significance between the indicated bar graph pairs; mean ± standard error of the mean (s.e.m.), n = 9 replicates from 3 independent experiments).

Fig 3. The ratio of optical densities at 450 nm versus 490 nm (OD450 nm/OD490 nm) for Aβ3(pE)–42 rose and peaked more rapidly than for Aβ1–42, but peaked at a ~25% lower level. The fastest rise in the
OD$_{450}$ nm/OD$_{490}$ nm ratio was for 5% pE-Aβ, which peaked similarly to Aβ$_{3(5pE)-42}$, Aβ$_{3gPE}-42$, Aβ$_{1-42}$ and 5% pE-Aβ thus oligomerized by different pathways.

To test whether distinct biological activities were coupled to these oligomerization differences, we compared cytotoxicity of the peptides towards cultured neurons or glia using calcein-AM and fluorescence microscopy.$^{16}$ Twelve hours of Aβ$_{1-42}$ exposure had little effect on cell viability for wild-type or tau-knockout neurons, or wild-type glial cells (Fig. 1a). Contrastingly, most wild-type neurons died and detached from the substrate after exposure to Aβ$_{1-42}$ or 5% pE-Aβ. Tau-knockout neurons and wild-type glia, which express little tau, were resistant to Aβ$_{1-42}$ and 5% pE-Aβ.

Cytotoxicity dose dependence was examined by incubating wild-type neurons for 24 h in oligomers comprising 0.1, 0.5 or 1 μM peptides, and using the 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay.$^{17}$ Cells were unaffected by Aβ$_{1-42}$, but Aβ$_{3(5pE)-42}$ and 5% pE-Aβ had substantial cytotoxicity at 0.5 μM and even more at 1.0 μM. Cytotoxicity of 5% pE-Aβ required Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ to incubate together for 24 h before being added to cells. When they were incubated separately for 24 h and mixed together at a 1:19 molar ratio immediately before being applied to cells, they were not cytotoxic. A small amount of Aβ$_{3(5pE)-42}$ can thus markedly enhance the cytotoxicity of a large excess of Aβ$_{1-42}$, provided the two peptides oligomerize together.

Evidence for hybrid oligomers came from immunoprecipitation of various forms of amyloid-β using aggregation-dependent M64, which does not recognize Aβ$_{3(5pE)-42}$ (see Supplementary Fig. 4 for characterization of all anti-amyloid-β antibodies used, including M64). Immunoprecipitations were analysed on dot blots using 4G8, which equally recognizes Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$, and anti-pE-Aβ, which does not react with Aβ$_{1-42}$. M64 immunoprecipitated oligomers made from Aβ$_{1-42}$ or 5% pE-Aβ, but it did not immunoprecipitate Aβ$_{3(5pE)-42}$ oligomers, nor monomers of either peptide (Fig. 2a). Because anti-pE-Aβ reacted with material immunoprecipitated out of 5% pE-Aβ, M64 pulled down hybrid peptide oligomers. Aβ$_{3(5pE)-42}$ accounted for ~16% of the amyloid-β in gel-filtered cytotoxic oligomers after 3 h of oligomerization, and steadily dropped to ~8% by 24 h (Fig. 2b). Aβ$_{3(5pE)-42}$ thus acts as a template that initiates formation of cytotoxic oligomers.

Cytotoxicity was sensitive to oligomerization time (Fig. 2c). Baseline cytotoxicity was observed at all time points for Aβ$_{1-42}$, and for 5% pE-Aβ solutions in which Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ oligomerized separately. Pure Aβ$_{3(5pE)-42}$ killed ~50% of the cells after 24 h of oligomerization, but was virtually non-toxic at 0 h and after 96 h of oligomerization. The most cytotoxic solutions were 5% pE-Aβ, in which the constituent peptides co-oligomerized for 24 h. These solutions killed ~60% of the cells within 24 h, and lower but robust cytotoxicity was observed at 96 h. Even the 0 h co-oligomers of 5% pE-Aβ exhibited low, significant cytotoxicity. Co-incubated mixtures of 5% Aβ$_{3(5pE)-42}$ and 95% Aβ$_{1-42}$, and 95% Aβ$_{1-42}$ and 5% pE-Aβ, were not cytotoxic.

Figure 2 | Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ form metastable, cytotoxic, hybrid oligomers. a, Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ were incubated together at a 1:19 molar ratio (5% pE-Aβ) for 24 h at 1 μM total amyloid-β, and were then immunoprecipitated (IP) with M64, a rabbit monoclonal antibody that specifically recognizes residues 3–7 (EFFFH) of Aβ$_{1-40}$ oligomers or fibrils. Additional samples that were immunoprecipitated included otherwise identically treated oligomers made from pure Aβ$_{3(5pE)-42}$ or Aβ$_{1-42}$ and monomeric versions of the two peptides. Immunoprecipitated oligomers were converted to monomers by lyophilization, solubilization with HFIP and dialysis into PBS, and along with the other samples were dot blotted onto nitrocellulose and analysed using 4G8, a mouse monoclonal antibody that recognizes Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ equally well, and an antibody that specifically recognizes pE-Aβ (see Supplementary Fig. 4 for characterization of all antibodies used here). Quantification of the dot blots using a LI-COR Odyssey imaging station indicated that the oligomers that were immunoprecipitated from the mixed peptide solution contained both Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$, at a molar ratio of ~1:10. b, Solutions containing 5% pE-Aβ-Aβ$_{3(5pE)-42}$ and 95% Aβ$_{1-42}$ were incubated for the indicated times, and then were fractionated by gel filtration. At each time point, fractions that eluted at 12.5 ml, where most cytotoxicity resided (see Fig. 3b) were immunoprecipitated using anti-human amyloid-β (N), an amino-terminal-specific antibody that does not react with pE-Aβ (data not shown). The immunoprecipitates were then lyophilized, solubilized with HFIP, and quantitatively analysed on dot blots with 4G8 and anti-pE-Aβ using the LI-COR Odyssey. The time-dependent decrease in the Aβ$_{3(5pE)-42}$ content of the immunoprecipitated oligomers implies that Aβ$_{3(5pE)-42}$ initiated formation of hybrid peptide oligomers. c, Aβ$_{3(5pE)-42}$ and Aβ$_{1-42}$ oligomerized for 0, 24 and 96 h either separately or together at 1:19 mixtures, and then were added to primary wild-type neuron cultures for 24 h at a final concentration of 1 μM total amyloid-β. Following peptide treatment, cell viability was analysed by the XTT plate reader assay.$^{17}$ The most cytotoxic species observed were the hybrid oligomers after 24 h of oligomerization (P < 0.01; yellow stars signify statistical significance of the indicated bar graphs versus vehicle controls; black stars signify statistical significance between the indicated bar graph pairs; mean ± s.e.m., n = 6 or 9 replicates from 3 independent experiments for panel b or c, respectively).
Aβ1-42 can therefore form oligomers whose cytotoxicity is both greater and more enduring than oligomers formed by Aβ3(3PE)-42 alone.

To identify the co-oligomer size(s) that were cytotoxic, amyloid-β solutions were oligomerized for various times from 0–96h before fractionation by gel filtration. Total amyloid-β in all fractions was determined using 4G8 dot blots that, as shown in Fig. 3a (for 5% pE-Aβ) and Supplementary Fig. 5 (for Aβ1-42 and Aβ3(3PE)-42), illustrate the full fractionation range of the column but exclude most void volume fractions. Presumptive monomeric Aβ1-42 gradually increased in size over the next 93 h. A fractionation by gel filtration. Total amyloid-solutions were oligomerized for various times from 0–96 h before

554 nM peptide for the larger oligomers that eluted at 8.5 ml. dimers/trimers that eluted at 12.5 ml, which at 425 nM peptide killed assayed for individual fractions of 5% pE-Aβ nearly 72 h for 5% pE-Aβ and more enduring than oligomers formed by Aβ1-42. These persisted as the main species for 24 h for Aβ1-42 both samples, when slightly larger species, LNOs that possibly corre-

ated differently. Putative monomers were present at 0 h for 

both samples, when slightly larger species, LNOs that possibly corre-

sponded to dimers or trimers (Supplementary Fig. 6), were also pre-

sent. These persisted as the main species for 24 h for Aβ3(3PE)-42 and for nearly 72 h for 5% pE-Aβ, and later time points were dominated by larger aggregates that eluted in void volume fractions. Cytotoxicity was assayed for individual fractions of 5% pE-Aβ that oligomerized for 24 h (Fig. 3b). Most cytotoxicity was associated with the possible dimers/trimers that eluted at 12.5 ml, which at 425 nm peptide killed more than 60% of the cells. Low cytotoxicity was also observed at 554 nM peptide for the larger oligomers that eluted at 8.5 ml.

The marked enhancement of Aβ1-42 cytotoxicity by Aβ3(3PE)-42 suggested a prion-like templating mechanism of Aβ1-42 misfolding initiated by Aβ3(3PE)-42. To test that hypothesis, 5% pE-Aβ that oligomerized for 24 h was diluted into 19 volumes of monomeric Aβ1-42. A 24 h incubation of this mixture yielded ‘serial passage 1’, which was followed by two equivalent, sequential dilutions into monomeric Aβ1-42 to yield serial passages 2 and 3. A gradual loss of cytotoxicity was observed with successive passages, but even passage 3, which contained only 0.000625% Aβ3(3PE)-42, killed ~50% of the neurons within 24 h (Fig. 3c). Serially passed gel-filtration samples contained abundant material that eluted at 12.5 ml in passages 1–3, despite the progressive dilution of Aβ3(3PE)-42 (Fig. 3d). Aβ3(3PE)-42 can therefore template formation of metastable, cytotoxic LNOs from excess Aβ1-42, yielding potent bioactivity that can be serially passed multiple times into monomeric Aβ1-42 without further addition of Aβ3(3PE)-42.

One possible explanation for why Aβ1-42 LNOs were inert is that they lacked sufficient properly sized oligomers. Accordingly, we altered the oligomerization protocol from 5 μM peptide for 24 h at 37 °C to 10 μM peptide for 30 min at 4 °C to obtain abundant Aβ1-42 oligomers that eluted at 12.5 ml (Fig. 3e). These LNOs were not cytotoxic (Fig. 3f), implying that they were structurally distinct from the putative dimers/trimmers initiated by Aβ3(3PE)-42. This was confirmed by dot blots using MB7, a conformation-sensitive anti-amyloid-β antibody, to compare the putative dimers/trimmers used for the cytotoxicity assays shown in Fig. 3f. We first lyophilized aliquots of all the amyloid-β solutions, resuspended them with hexafluoroisopropanol (HFIP) to restore them to monomers, and then analysed them using 4G8. When parallel samples that were not lyophilized but were otherwise identical were analysed using MB87, immunoreactivity was approximately twice as strong with LNOs made from Aβ1-42 versus those made from 5% pE-Aβ (Supplementary Fig. 7). Cytotoxic LNOs of 5% pE-Aβ are thus structurally distinct from comparably sized LNOs of Aβ1-42.

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month-old TBA2.1 mice generating Aβ3(PE)x,22 show amyloid-β deposits (arrows), massive astrogliosis (GFAP) and neuron loss (Hem., haematoxylin nuclear staining), none of which are evident in comparably aged wild-type (WT) mice or TBA2.1/tau-knockout19 (KO) hybrids.

Figure 4 | Aβ3(PE)x in vivo. a, Cytosol obtained from human Alzheimer’s disease (AD) and similarly aged normal brains (Supplementary Fig. 9) were fractionated by gel filtration, and analysed by dot blotting with anti-pE-Aβ and M87. Note the appearance of pE-Aβ in LNO fractions, including those that eluted at 12.4 ml, especially in the Alzheimer’s disease samples. b, Three-

Several lines of evidence demonstrate in vivo relevance for the data described so far. First, we identified LNOs containing Aβ3(PE)x in three out of three Alzheimer’s disease samples, based on gel filtration of human brain extracts followed by dot blots of resulting fractions with anti-pE-Aβ and M87. In contrast, only one of three age-matched samples with normal neuropathological diagnoses was positive for Aβ3(PE)x (Fig. 4a and Supplementary Fig. 8). Second, we crossed TBA2.1 mice accumulated small amounts (40–100 ng g⁻¹ brain weight) of Aβ3(PE)x, which formed primarily intraneuronal aggregates, and was associated with massive hippocampal neuron loss and gliosis18. Knocking out tau provided almost complete protection against neuron loss and glial activation (Fig. 4b). Additional in vivo data are shown in Supplementary Fig. 9. Long-term potentiation (LTP) of mouse hippocampal neurons in slice cultures was potently and equally inhibited by oligomers made from 5% Aβ3(PE)x or 100% Aβ3(PE)x, whereas Aβ3(PE)x oligomers had no effect on LTP. 1% Aβ3(PE)x provoked mild, but statistically insignificant LTP impairment (Supplementary Fig. 9a). To evaluate the effects of increased Aβ3(PE)x in animal models, we crossed mice with neuron-specific expression of human β-amyloid precursor protein (APP) harbouring Swedish and London mutations (hAPPsw)35, with mice expressing human QC21. Nine-month-old double (hAPPsw/hQC) and single (hAPPsw) transgenic mice were indistinguishable in terms of insoluble and soluble Aβ3(PE)x levels, but the double transgenics had approximately twofold more insoluble Aβ3(PE)x and approximately ninefold more soluble Aβ3(PE)x than single transgenics (Supplementary Fig. 9b). Further analysis of the soluble Aβ3(PE)x by the A4 assay22 revealed an approximately eightfold excess of oligomers in the double versus single transgenics (Supplementary Fig. 9c). Double transgenics performed more poorly in Morris water maze tests (Supplementary Fig. 9d) and had reduced hippocampal immuno-reactivity for the synapse marker, synaptophysin (Supplementary Fig. 9e). Finally, perihippocampal injection of 5% pE-Aβ at 5 μM into APPsw/hQC NOS2⁻/⁻/Alzheimer’s disease model mice23 led 3–5 months later to the presence of plaques containing both pE-Aβ and conventional amyloid-β. Comparable plaques were rarely seen in sham-injected Alzheimer’s disease mice or in wild-type mice injected with 5% pE-Aβ (Supplementary Fig. 9f). These collective in vivo results emphasize the physiological significance of the companion biochemical and cultured cell results.

Our studies provide new insights into Alzheimer’s disease pathogenesis by demonstrating that hypertoxic amyloid-β oligomers can be triggered by small quantities of a specifically truncated and post-translationally modified version of amyloid-β. Although some previous studies demonstrated that pE modification of amyloid-β considerably enhances its aggregation kinetics13,14,24, toxicity12,18,25 and resistance to degradation18, a mechanistic explanation for the unique properties of pE-Aβ has been lacking until now. Prior studies suggest coincident appearance of Aβ3(PE)x with development or progression of human Alzheimer’s disease26,27. Co-localization of QC and Aβ3(PE)x was found in cored plaques of vulnerable regions in Alzheimer’s disease, and evidence was provided for axonal transport of Aβ3(PE)x from QC-rich neuronal populations of the entorhinal cortex and locus coeruleus28. As LNOs containing Aβ3(PE)x are reasonably stable (Fig. 3a), they might initiate tau-dependent cytotoxicity intracellularly during axonal transport29 or extracellularly following release at remote hippocampal synapses30 of projection neurons18. The Aβ3(PE)x-induced formation of toxic mixed oligomers provides a rationale for these previous observations, and the tau-dependent cytotoxicity of 5% pE-Aβ establishes a new functional connection between amyloid-β and tau in Alzheimer’s disease pathogenesis.

METHODS SUMMARY

Full descriptions of thioflavin T assays, cell culture, cell viability assays, procedures for oligomerization of amyloid-β peptides and their fractionation by gel-filtration chromatography, production and specificity of rabbit monoclonal anti-amyloid-β antibodies, immunoprecipitation, dot blots and western blots, generation of hAPPsw/hQC transgenic mice, LTP measurements of mouse hippocampal slice cultures, peri-hippocampal injection of 5% pE-Aβ into Alzheimer’s disease model mice, cultured cell and brain immunohistochemistry, and collection of human brain extracts are provided in Supplementary Methods.
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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.