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Pyroglutamate-Aβ 3 and 11 colocalize in amyloid plaques in Alzheimer's disease cerebral cortex with pyroglutamate-Aβ 11 forming the central core

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ABSTRACT

N-terminal truncated amyloid beta (Aβ) derivatives, especially the forms having pyroglutamate at the 3 position (AβpE3) or at the 11 position (AβpE11) have become the topic of considerable study. AβpE3 is known to make up a substantial portion of the Aβ species in senile plaques while AβpE11 has received less attention. We have generated very specific polyclonal antibodies against both species. Each antibody recognizes only the antigen against which it was generated on Western blots and neither recognizes full length Aβ. Both anti-AβpE3 and anti-AβpE11 stain senile plaques specifically in Alzheimer's disease cerebral cortex and colocalize with Aβ, as shown by confocal microscopy. In a majority of plaques examined, AβpE11 was observed to be the dominant form in the innermost core. These data suggest that AβpE11 may serve as a generating site for senile plaque formation.

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During the last twenty-five years, much evidence has linked the onset of Alzheimer's disease (AD) to the accumulation of a variety of forms of the amyloid beta (Aβ) peptide [11]. Full-length Aβ (amino acid residues 1–40 and 1–42) has been the dominant foci of research, but amino (N) and carboxy-terminally truncated as well as modified, forms of Aβ also exist. When N-terminal truncation exposes a glutamic acid residue, the amino terminus of Aβ can become pyrolyzed forming a stable ring [3]. One of these post-translationally modified forms of Aβ, pyrolyzed Aβ3-x (AβpE3), is abundant in brain regions affected in AD [4,8,9,15,21,22]. A second form of pyrolyzed Aβ, Aβ11-x (AβpE11) has received less attention, but also colocalizes with Aβ1–40/42 containing plaques in AD brain [7,12]. This presence of AβpE3 and AβpE11 peptides in AD brains is in contrast to full length forms of Aβ that predominate in non-demented elderly control brain tissue [7,13,22]. In brain tissue from subjects with Down's syndrome, pathologically similar to that of AD [10], AβpE11 has been identified even before birth [7]. How the various N terminally truncated species of Aβ, as well as the post-translationally modified derivatives of these species, are generated, and how they contribute to neurodegeneration, are currently the subject of intense research [3].

Studies thus far indicate that generation of AβpE3 is a multi-step process. The first two N-terminal amino acids of Aβ are sequentially cleaved intracellularly by aminopeptidase A [19]. This cleavage is then followed by the pyrolysis of the resulting N-terminal glutamic acid, producing AβpE3 thus rendering it more resistant to further degradation. Cloning of the β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE 1) has demonstrated that AβE11 can be generated directly following BACE-1 cleavage of APP [20] followed by γ-secretase cleavage. Additionally, the major proteolytic product of APP, C99, can also produce AβE11 through sequential cleavage by BACE 1 and γ-secretase [6].

Production *in vitro* of AβpE3 and AβpE11 is extremely slow but glutaminyl cyclase (QC) in the brain, predominantly localized in the Golgi apparatus [1], rapidly catalyzes conversion of AβE3 to form AβpE3. QC also catalyzes conversion of AβE11 to AβpE11 [18]. AβpE rapidly adopts a β-sheet conformation and is significantly more toxic and stable than unmodified, full length Aβ [2,14,16].

Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's disease; N, amino; APP, amyloid precursor protein; BACE 1, beta-site amyloid precursor protein cleaving enzyme 1; QC, glutaminyl cyclase; HPLC, high pressure liquid chromatography; KO, knock out; AβpE3, pyrolyzed Aβ3-x; AβpE11, pyrolyzed Aβ11-x.

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Recent studies demonstrate increased AβpE3 levels and early accumulation of AβpE3 oligomers in neurons in a transgenic mouse model for AD and in neurons of patients with AD [21]. Passive immunization of the transgenic mice with an antibody that selectively recognizes oligomeric assemblies of AβpE3 not only reduced AβpE3 levels but also normalized behavioral deficits [22]. Moreover, when the transgenic mouse model with abundant AβE3 formation, was crossed with transgenic mice expressing human QC (hQC), the brain tissue from their bigenic progeny showed significant elevation in soluble and insoluble AβpE3 peptides and greater amounts of AβpE3 in plaques. When 6-months old, these bigenic mice also had significant motor and working memory impairment compared to non-hQC transgenic mice. The contribution of endogenous mouse QC (mQC) was examined by then knocking out mQC in the single transgenic AD mouse model. The mQC-KO mice showed significant rescue of wild-type mouse behavioral phenotype [5]. In the same transgenic mouse line, pharmacological inhibition of QC activity produced the same effects as QC KO [17]. The collective data from these studies strongly support the notion that a AβpE peptide(s) plays a key role in the neuropathology of AD.

To date, there are no studies which have simultaneously examined the deposition and spatial localization of AβpE3 and AβpE11 in brain tissue from AD subjects. To perform these studies, we have generated antibodies that recognize AβpE3 and AβpE11 specifically and used these reagents to elucidate the spatial relationship of AβpE3 and AβpE11 to one another, as well as to Aβ in AD brain tissue. Here we find that both AβpE3 and 11 are abundant in amyloid plaques and that they colocalize with each other as well as with Aβ. Additionally, we noted a higher level of AβpE11 in plaque cores when compared with AβpE3 and full length Aβ species. This observation supports the hypothesis that AβpE11 may be an early aggregating form of Aβ and thus act as the seed or nidus for senile plaque formation.

All Aβ peptides were synthesized using standard Fluorenylmethyloxycarbonyl chemistry. Synthesized peptides are cleaved off the resin using trifluoroacetic acid (TFA). Crude peptides were purified using preparative reversed-phase high pressure liquid chromatography (HPLC) using a water and acetonitrile with 0.1 TFA for elution. Peptides were purified to >85% purity as determined by analytical HPLC.

Purified peptides were conjugated to a proprietary mix of carrier proteins (21st Century Biochemicals) and injected into New Zealand white rabbits (Cocalico Farms). A NIH-approved protocol was followed and includes both complete and partial *Freund's* adjuvant with 7 full production bleeds followed by a two-part exsanguination.

Antibodies were purified using peptides linked to iodoacetyl agarose. Sera showing high antigen reactivity by ELISA against AβpE3–11 or AβpE11–19 respectively, were pooled and extensively immunodepleted with full length and truncated Aβ peptides. Depleted sera were then purified using either AβpE3–11 or AβpE11–19 and eluted using glycine. Eluates were neutralized and then dialyzed.

SDS–PAGE was carried out using 10–20% or 15% Tris/Glycine gels (Biorad) followed by transfer onto a 0.2 μm PVDF membrane (Whatman). A slot blot apparatus (Immunetics, Boston, MA) was used to probe duplicate lanes with varying concentrations of antibody. Antibody specificity and sensitivity were detected using enhanced chemiluminescence.

Double and triple immunofluorescence staining were carried out to assess colocalization by standard fluorescence microscopy or confocal imaging using a Leica SP5 laser scanning confocal microscope. 5 μm paraffin embedded tissue sections from frontal cortex were obtained through the VA VISN1 Neuropathology Center. Sections were de-paraffinized in xylene and rehydrated through alcohol before being subjected to antigen retrieval in formic acid. Sections were blocked in Super Block buffer (ScyTek) containing 5% normal donkey serum. Primary antibodies [including anti-AβpE3 (21st Century Biochemicals, used at 0.5 μg/ml) or AβpE11 (21st Century Biochemicals, used at 0.5 μg/ml) Cy3-tagged anti-AβpE3 (21st Century Biochemicals), Cy5-tagged anti-AβpE11 (21st Century Biochemicals) and anti-Aβ (Dako, used at 0.5 μg/ml)] were incubated with tissue sections overnight at 4 °C. Where appropriate, sections were then incubated for 1 h at room temperature with a biotinylated secondary antibody (2 μg/ml) followed by avidin particles conjugated with either horseradish peroxidase (HRP) (ABC Detection System, Vector Labs), Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen, used at 15 μg/ml). This protocol (beginning with blocking procedures) was repeated for fluorescent secondary antigen detection in sequential staining procedures. Avidin HRP was detected by treating sections for 10 min with 3,5-diaminobenzoic acid (DAB) to yield a brown precipitate.

Rehydrated sections were stained with 1% Thioflavin S solution for 10 min. Stained sections were then dehydrated and rinsed in distilled water before mounting with aqueous mounting medium.

To visualize AβpE in brain tissue sections, antibodies were developed against AβpE3 and AβpE11. The sensitivity of these antibodies for protein detection was analyzed by Western blot (Supplementary data figure* S1). AβpE3–42 (loaded at ~10 ng/lane) was run on a 2D, 10–20% Tris/Tricine gel and immunoblotted with anti-AβpE3 antibody. Figure S1 shows that as little as 5 ng/ml of the antibody can detect AβpE3–42. Similar results were obtained with the anti-AβpE11 antibody (Figure S2). Blocking experiments demonstrated specificity of both anti-AβpE3 and anti-AβpE11 antibodies, with complete blocking using the peptide to which the antibody was produced and no blocking with Aβ1–40, Aβ3–9, or Aβ11–19 (data not shown). The two anti-AβpE antibodies also showed no cross-reactivity with one another (data not shown).

Previous studies have reported the presence of AβpE species in Aβ plaques [7,15]. To assess the colocalization of AβpE3 and AβpE11 with Aβ plaques using our antibodies, human frontal cortex tissue sections were double immunostained and analyzed by confocal microscopy. Fig. 1 is representative of tissue sections labeled with an anti-Aβ antibody (A,D,G,J,M,P) together with either anti-AβpE3 (B,H,N) or anti-AβpE11 antibodies (E,K,Q). Both anti-AβpE3 and anti-AβpE11 are specifically blocked only by the peptide against which they were produced (K and N, respectively), and are not blocked by Aβ1–40 (data not shown) or by a non-target peptide (H and Q). Both AβpE3 and 11 were found within Aβ containing plaques. The merged images show areas of colocalization (yellow) between Aβ and AβpE3 (C,I,O) or AβpE11 (F,L,R). Interestingly, AβpE11 was found to be present most heavily in plaque cores, while AβpE3 was observed to colocalize more completely with Aβ. No staining with either anti-AβpE3 or anti-AβpE11 was seen in the same brain region of a 23 year old subject (data not shown).

To ascertain that the AβpE3 and AβpE11 containing plaques were composed of amyloid fibers, we localized each pyrolated species together with Thioflavin S, a compound that can be detected by fluorescence microscopy when bound to amyloid fibers. Fig. 2 shows that both AβpE3 and AβpE11 colocalize with plaques labeled by Thioflavin S staining.

Although AβpE species have been shown individually to be present in amyloid plaques, their colocalization with each other has not been studied. To determine whether or not AβpE3 and AβpE11 colocalize in amyloid plaques, we used confocal microscopy employing purified fluorescently tagged primary antibodies against each derivative. We also employed a mouse monoclonal antibody against Aβ, which was detected in subsequent steps using a third fluorescent probe. All three primary antibodies were added simultaneously at the same concentration.

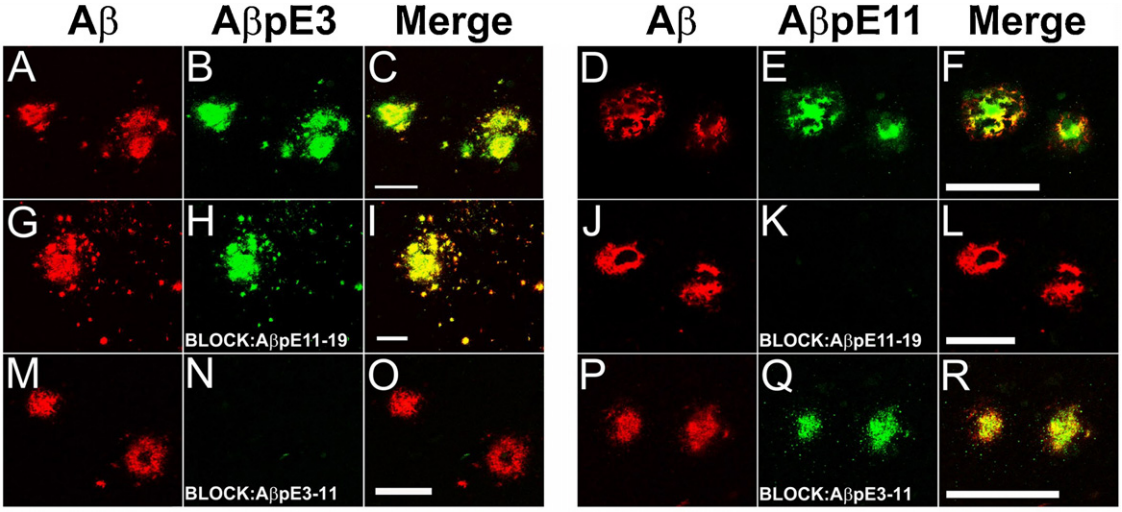

Fig. 1. Aβ colocalizes with AβpE3 and AβpE11 in the frontal cortex. Confocal microscopy was carried out to assess colocalization of AβpE species with Aβ plaques. Human brain sections were subjected to immunohistochemistry with antibodies against AβpE3 (green, B,H,N), AβpE11 (green, E,K,Q), and Aβ (red, A,D,G,J,M,P). Specificity of antibodies to AβpE3 and AβpE11 was confirmed by pre-incubation of each with their target peptide (N or K respectively) or non-target peptide as control (H and Q, respectively). Areas of colocalization of AβpE3 and Aβ (C,I,O) and of AβpE11 and Aβ (F,L,R) appear yellow in merged panels. White scale bars are 50 μm in length. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

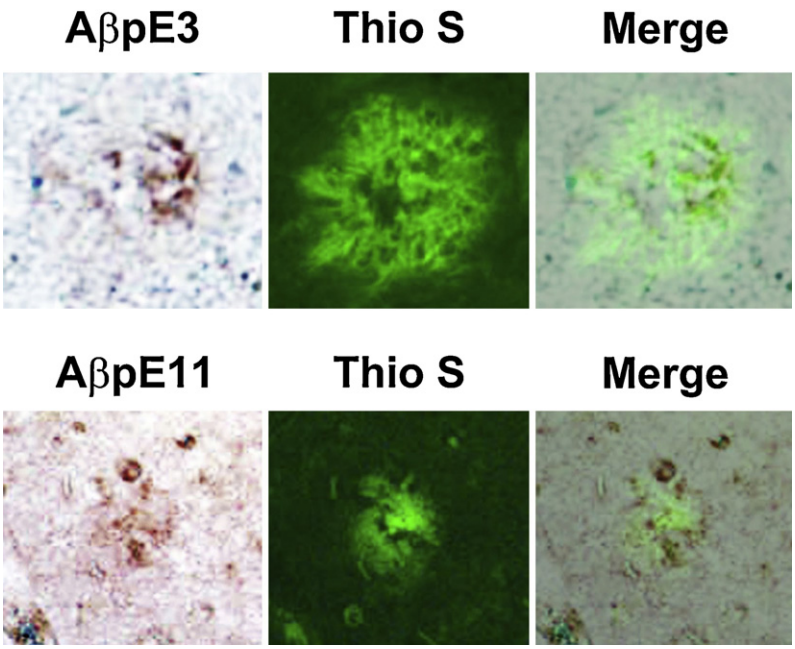

Fig. 2. AβpE3 and AβpE11 are present in senile plaques. Sections from the frontal cortex of AD patients were stained with Thioflavin S dye (green, binds to β-pleated amyloid fibrils found within mature senile plaques). Immunostaining was also performed on the same sections using antibodies against AβpE3 (brown, top) and AβpE11 (brown, bottom). Overlap of AβpE3 and AβpE11 with β-pleated amyloid fibrils was visualized by merging the thioflavin stained and immunostained images.

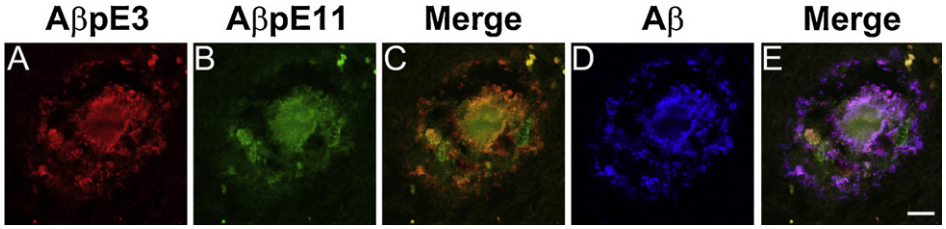

Fig. 3. AβpE3 colocalizes with AβpE11 in Aβ plaques. Triple immunostaining was carried out using antibodies against AβpE3 (A), AβpE11 (B) and Aβ (D). Samples were examined by confocal microscopy for colocalization of AβpE3 and AβpE11 (C) and for colocalization of AβpE3, AβpE11, and Aβ (E). White scale bar is 50 μm.

A representative plaque (Fig. 3) demonstrates that both pyrolated Aβ species are present in an Aβ containing plaque. In order to determine what percentage of plaques contain each of the three Aβ species examined, 20 plaques were identified in each of 5 AD cerebral cortex samples using the same triple fluorescent labeling method. All three Aβ species were observed in each of the plaques examined. Although all three antigens colocalize in much of the plaque, of particular interest (and in agreement with our previous observation) is the observation that AβpE11 is the dominant form of Aβ at the core of the plaque.

In the present study, we generated two polyclonal antibodies against AβpE species and used them to observe the AβpE profile of human amyloid plaques. Evaluation of these antibodies by Western blot analysis and immunohistochemistry demonstrated that they bind specifically to their targets at low concentrations (Figures S1, S2 and 1).

In AD brain, AβpE3 and 11 were found in abundance in mature senile plaques (Fig. 2) where they colocalize with full length Aβ as well as with each other (Fig. 3). In these plaques, we consistently observed AβpE11 rich cores. This was not true of every plaque observed; however, with the use of 5 μm tissue sections, many of the plaques observed are not centrally cross sectioned. That being said, AβpE11 core plaques were prominent in the tissue sections observed. Future experiments using thicker tissue sections will strengthen the argument for or against AβpE11 cores. We hypothesize that intracellularly generated AβpE11 aggregates inside neurons, possibly into an oligomeric species as has been shown with AβpE3 [22]. We hope to determine whether intraneuronal AβpE11 is present at an early stage of AD pathogenesis in future investigations. We also hypothesize that aggregated AβpE11 enters the extracellular space either after cell death or by some other mechanism (e.g., secretion) and serves as seeds for the formation of extracellular amyloid plaques. Further, we predict that the amount of AβpE11 in the CSF and blood may be an early and specific detector of presymptomatic AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.09.071.

References

[1] H. Cynis, E. Scheel, T.C. Saido, S. Schilling, H.U. Demuth, Amyloidogenic processing of amyloid precursor protein: evidence of a pivotal role of glutaminyl cyclase in generation of pyroglutamate-modified amyloid-beta, Biochemistry 47 (2008) 7405–7413.
[2] C. D'Arrigo, M. Tabaton, A. Perico, N-terminal truncated pyroglutamyl beta amyloid peptide Abetapy3–42 shows a faster aggregation kinetics than the full-length Abeta1–42, Biopolymers 91 (2009) 861–873.
[3] A.P. Gunn, C.L. Masters, R.A. Cherny, Pyroglutamate-Abeta: role in the natural history of Alzheimer's disease, Int. J. Biochem. Cell Biol. 42 (2010) 1915–1918.
[4] M. Hartlage-Rubsamen, M. Morawski, A. Waniek, C. Jager, U. Zeitschel, B. Koch, H. Cynis, S. Schilling, R. Schliebs, H.U. Demuth, S. Rossner, Glutaminyl cyclase contributes to the formation of focal and diffuse pyroglutamate (pGlu)-Abeta deposits in hippocampus via distinct cellular mechanisms, Acta Neuropathol. 121 (2011) 705–719.
[5] S. Jawhar, O. Wirths, S. Schilling, S. Graubner, H.U. Demuth, T.A. Bayer, Overexpression of glutaminyl cyclase, the enzyme responsible for pyroglutamate A{beta} formation, induces behavioral deficits, and glutaminyl cyclase knockout rescues the behavioral phenotype in 5XFAD mice, J. Biol. Chem. 286 (2010) 4454–4460.
[6] K. Liu, R.W. Doms, V.M. Lee, Glu11 site cleavage and N-terminally truncated A beta production upon BACE overexpression, Biochemistry 41 (2002) 3128–3136.
[7] K. Liu, I. Solano, D. Mann, C. Lemere, M. Mercken, J.Q. Trojanowski, V.M. Lee, Characterization of Abeta11–40/42 peptide deposition in Alzheimer's disease and young Down's syndrome brains: implication of N-terminally truncated Abeta species in the pathogenesis of Alzheimer's disease, Acta Neuropathol. 112 (2006) 163–174.
[8] M. Morawski, M. Hartlage-Rubsamen, C. Jager, A. Waniek, S. Schilling, C. Schwab, P.L. McGeer, T. Arendt, H.U. Demuth, S. Rossner, Distinct glutaminyl cyclase expression in Edinger-Westphal nucleus, locus coeruleus and nucleus basalis Meynert contributes to pGlu-Abeta pathology in Alzheimer's disease, Acta Neuropathol. 120 (2010) 195–207.
[9] H. Mori, K. Takio, M. Ogawara, D.J. Selkoe, Mass spectrometry of purified amyloid beta protein in Alzheimer's disease, J. Biol. Chem. 267 (1992) 17082–17086.
[10] R.E. Mrak, W.S. Griffin, Trisomy 21 and the brain, J. Neuropathol. Exp. Neurol. 63 (2004) 679–685.
[11] M.P. Murphy, H. LeVine 3rd, Alzheimer's disease and the amyloid-beta peptide, J. Alzheimers Dis. 19 (2010) 311–323.
[12] R. Perez-Garmendia, V. Ibarra-Bracamontes, V. Vasilevko, J. Luna-Munoz, R. Mena, T. Govezensky, G. Acero, K. Manoutcharian, D.H. Cribbs, G. Gevorkian, Anti-11[E]-pyroglutamate-modified amyloid beta antibodies cross-react with other pathological Abeta species: relevance for immunotherapy, J. Neuroimmunol. 229 (2010) 248–255.
[13] A. Piccini, C. Russo, A. Gliozzi, A. Relini, A. Vitali, R. Borghi, L. Giliberto, A. Armirotti, C. D'Arrigo, A. Bachi, A. Cattaneo, C. Canale, S. Torrassa, T.C. Saido, W. Markesbery, P. Gambetti, M. Tabaton, beta-Amyloid is different in normal aging and in Alzheimer disease, J. Biol. Chem. 280 (2005) 34186–34192.
[14] C.J. Pike, M.J. Overman, C.W. Cotman, Amino-terminal deletions enhance aggregation of beta-amyloid peptides in vitro, J. Biol. Chem. 270 (1995) 23895–23898.
[15] T.C. Saido, T. Iwatsubo, D.M. Mann, H. Shimada, Y. Ihara, S. Kawashima, Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques, Neuron 14 (1995) 457–466.
[16] S. Schilling, T. Lauber, M. Schaupp, S. Manhart, E. Scheel, G. Bohm, H.U. Demuth, On the seeding and oligomerization of pGlu-amyloid peptides (in vitro), Biochemistry 45 (2006) 12393–12399.
[17] S. Schilling, U. Zeitschel, T. Hoffmann, U. Heiser, M. Francke, A. Kehlen, M. Holzer, B. Hutter-Paier, M. Prokesch, M. Windisch, W. Jagla, D. Schlenzig, C. Lindner, T. Rudolph, G. Reuter, H. Cynis, D. Montag, H.U. Demuth, S. Rossner, Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology, Nat. Med. 14 (2008) 1106–1111.
[18] F. Seifert, K. Schulz, B. Koch, S. Manhart, H.U. Demuth, S. Schilling, Glutaminyl cyclases display significant catalytic proficiency for glutamyl substrates, Biochemistry 48 (2009) 11831–11833.
[19] J. Sevalle, A. Amoyel, P. Robert, M.C. Fournie-Zaluski, B. Roques, F. Checler, Aminopeptidase A contributes to the N-terminal truncation of amyloid beta-peptide, J. Neurochem. 109 (2009) 248–256.
[20] R. Vassar, B.D. Bennett, S. Babu-Khan, S. Kahn, E.A. Mendiaz, P. Denis, D.B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M.A. Jarosinski, A.L. Biere, E. Curran, T. Burgess, J.C. Louis, F. Collins, J. Treanor, G. Rogers, M. Citron, Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, Science 286 (1999) 735–741.
[21] O. Wirths, T. Bethge, A. Marcello, A. Harmeier, S. Jawhar, P.J. Lucassen, G. Multhaup, D.L. Brody, T. Esparza, M. Ingelsson, H. Kalimo, L. Lannfelt, T.A. Bayer, Pyroglutamate Abeta pathology in APP/PS1KI mice, sporadic and familial Alzheimer's disease cases, J. Neural Transm. 117 (2010) 85–96.
[22] O. Wirths, C. Erck, H. Martens, A. Harmeier, C. Geumann, S. Jawhar, S. Kumar, G. Multhaup, J. Walter, M. Ingelsson, M. Degerman-Gunnarsson, H. Kalimo, I. Huitinga, L. Lannfelt, T.A. Bayer, Identification of low molecular weight pyroglutamate A{beta} oligomers in Alzheimer disease: a novel tool for therapy and diagnosis, J. Biol. Chem. 285 (2010) 41517–41524.