Pyroglutamate Amyloid-β (Aβ): A Hatchet Man in Alzheimer Disease*

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Pyroglutamate-modified amyloid-β (Aβ\textsubscript{pE3}) peptides are gaining considerable attention as potential key participants in the pathology of Alzheimer disease (AD) due to their abundance in AD brain, high aggregation propensity, stability, and cellular toxicity. Transgenic mice that produce high levels of Aβ\textsubscript{pE3-42} show severe neuron loss. Recent in vitro and in vivo experiments have proven that the enzyme glutaminyl cyclase catalyzes the formation of Aβ\textsubscript{pE3}. In this minireview, we summarize the current knowledge on Aβ\textsubscript{pE3}, discussing its discovery, biochemical properties, molecular events determining formation, prevalence in the brains of AD patients, Alzheimer mouse models, and potential as a target for therapy and as a diagnostic marker.

When Alois Alzheimer presented the case of his patient Auguste Deter at the Tübingen meeting of the Southwest German Psychiatrists in 1906, he did not attract much attention or stimulate any discussion in the audience. The young doctor likely would not have believed that, 100 years later, the disease that now holds his name would be the most common cause of dementia and a source of a critical medical and economical problem. At this meeting, Alzheimer presented Auguste Deter’s symptoms and reported the histopathological features that are now associated with Alzheimer disease (AD)\textsuperscript{2}: neuron loss, extracellular amyloid plaques, and intracellular neurofibrillary tangles. For more than 2 decades, the amyloid hypothesis has been the cardinal hypothesis in describing the sequence of AD etiology. The amyloid hypothesis considers amyloid-β (Aβ) deposition to be the causative event of AD pathology and that neurofibrillary tangles, cell loss, vascular damage, and dementia occur as a consequence of it (1). However, it has been recently suggested that the extracellular formation of Aβ plaques and other AD pathological events are preceded by intraneuronal Aβ accumulation, giving rise to a modified amyloid hypothesis (2).

The story of successful discoveries in modern AD research using novel molecular biological tools started with the biochemical analysis of β-amyloid-containing blood vessels (containing amyloid angiopathy) (3) and amyloid plaques consisting of Aβ (4), which led to the isolation and sequencing of the gene encoding the larger amyloid precursor protein (APP) (5, 6).

In vitro and in vivo analyses of amyloid deposits in AD revealed various N- and C-terminal variants (4, 7, 8). Increased C-terminal length of Aβ (from Aβ\textsubscript{40-42} to Aβ\textsubscript{42-42}) in AD enhanced aggregation and early deposition and promoted the toxicity of Aβ (9–11). Recently, Aβ\textsubscript{41-43} has been discussed as a novel toxic peptide in AD (12).

Beside Aβ peptides, starting with aspartate as the first amino acid (Aβ\textsubscript{7-1}), several N-terminally truncated and modified Aβ species have been described (4, 13–15). Among Aβ species present in AD plaques, Lewis et al. (16) reported that Aβ\textsubscript{3-42} is a relatively abundant species in AD, aged control, and vascular dementia patients. Using immunoprecipitation in combination with mass spectrometry, Portelius et al. (17) showed that Aβ\textsubscript{40} Aβ\textsubscript{41-42} Aβ\textsubscript{pE3-42} and Aβ\textsubscript{42-42} are the dominant fractions in AD hippocampus and cortex. Interestingly, it has been demonstrated that N-terminal deletions enhance Aβ aggregation by comparing Aβ\textsubscript{40-42} with Aβ\textsubscript{41-42} (9).

In addition, biochemical studies showed that Aβ peptides isolated from AD brains were post-translationally modified by isomerization and racemization (18, 19). Aβ isomerized at the seventh amino acid was suggested to compose a major fraction (Aβ\textsubscript{7-1}) (20). Both modifications have been shown to accelerate peptide aggregation and fibril formation (18, 21, 22). Other modifications include metal-induced oxidation (23) or phosphorylation (24, 25). In general, N-terminal modifications significantly influence the Cu\textsuperscript{2+} coordination of Aβ, which may be critical for alterations in aggregation propensity, redox activity, and resilience to degradation (26). In addition, it has been shown that certain apolipoprotein E isoforms bind to Aβ\textsubscript{1-42} and with less avidity to modified species, including isomerized and pyroglutamate-modified Aβ (Aβ\textsubscript{pE3}) (27). The controversy among different studies regarding the predominant species and their contribution to the pathology of AD might reflect differences in the brain regions analyzed, imbalances in age and disease stages of the recruited cases, different protocols utilized, and the characteristics of the peptides under investigation.

Discovery of Pyroglutamate Aβ

To unravel the pathogenic properties of Aβ, it was important to develop approaches to extract and study the biochemical nature of Aβ. Limited extraction and sequencing methods rendered it impossible for a long time. Attempts to extract and study the composition of Aβ plaques from brains of AD patients started in the 1970s (28, 29). However, discrepancies existed between different reports describing the amino acid sequence of Aβ due to different purification and analytical procedures. The first successful protocol to purify and study the sequence of amino acids of Aβ was developed by Glenner and Wong (30). Using collagenase treatment, they characterized Aβ from cerebrovascular amyloid in AD and Down syndrome (DS)
patients. They reported the sequence of the first 24 amino acids in Aβ, showing similarity between AD and DS patients. In a protocol that involved overnight digestion with pepsin, Masters et al. (4) reported the presence of ragged N termini of Aβ extracted from the plaque cores of AD and DS patients. According to them, 64% of the total Aβ peptides started with phenylalanine at position 4. Soon after that, Selkoe et al. (31) reported that they were not able to obtain N-terminal sequences from plaques using purification with SDS-containing buffer without protease treatment. Thus, they suggested that the N terminus of Aβ might be blocked (31). In line with this, other teams did not succeed in obtaining interpretable N-terminal sequences from plaque cores isolated by other methods (32). Finally, this discrepancy was solved by Mori et al. (33), who described the presence of Aβ peptides (15–20% of the total Aβ) bearing a pyroglutamate residue at the N terminus. By using pyroglutamylaminopeptidase, they were able to unravel the amino acid N terminus, which is blocked by the lactam ring and thus resistant to any other peptidase for Edman sequencing used in previous reports (33).

Since then, the interest in dissecting the temporal and spatial deposition of pyroglutamyl Aβ has increased. Many techniques and protocols were developed to increase the sensitivity to different forms of Aβ, especially the Aβ\textsubscript{pe3} peptides (34, 35). Equipped with a set of novel antibodies, Saida et al. (15) showed by immunohistochemical and biochemical means that Aβ\textsubscript{pe3} is present in equivalent or larger amounts than full-length Aβ in senile plaques. On the basis of analysis of brain tissue from DS cases, the authors also suggested that Aβ\textsubscript{pe3–3} precedes the deposition of unmodified Aβ (Aβ\textsubscript{1–3}). However, a study on the sequential deposition of heterogeneous forms of Aβ in the brains of DS patients could not detect Aβ\textsubscript{pe3} in young patients. Nevertheless, in agreement with the results of Saida et al., Aβ\textsubscript{pe3} always exceeded the deposition of Aβ\textsubscript{1} (36). This was further confirmed by another study on water-soluble Aβ demonstrating the presence of Aβ\textsubscript{pe3–42} in AD and DS patients as a dominant fraction (37). In line with the previous findings, testing extracts from AD and DS frontal cortex using ELISA revealed that levels of Aβ\textsubscript{pe3} and isomerized Aβ species ending at amino acid 42 were higher than those ending at amino acid 40 (38, 39). This was further confirmed by the finding that Aβ\textsubscript{pe3–42} constituted 25% of the total Aβ\textsubscript{1–42} in plaques of AD brains (39). It was reported that unmodified Aβ\textsubscript{1–40} and Aβ\textsubscript{1–42} can be modified into Aβ\textsubscript{pe3} after being injected into rat brain, indicating that rat brains harbor the enzymes required for N-terminal truncation and pyroglutamyl formation (40).

N-terminal Truncation Is a Prerequisite of Pyroglutamyl Aβ

Formation of pyroglutamyl-modified Aβ is a multistep process requiring the removal of the first two amino acids, aspartate and alanine, to expose the N-terminal glutamate at the third position of Aβ (Fig. 1). After cleavage of APP by the major β-site APP-cleaving enzyme (BACE1) and γ-secretase, Aβ\textsubscript{1–40/42} is liberated. Data from our group suggest that the levels of Aβ\textsubscript{pe3, x} are inversely linked to the levels of Aβ\textsubscript{1–3} in plaques in APP/PS1KI transgenic mice (41). In line with this observation, Sevall et al. (42) suggested that aminopeptidase A can trigger the initial first amino acid (aspartate) cleavage of Aβ. Interestingly, more than 15 years ago, Saida et al. (15) sug-

**FIGURE 1. Generation of pyroglutamyl Aβ.** The first N-terminal two amino acids, aspartate and alanine, are cleaved off by an unknown mechanism, exposing glutamate at position 3 of the N terminus of Aβ. Subsequently, glutamate is post-translationally modified to N-terminal pyroglutamate (pE) by dehydration catalyzed by QC activity. The novel peptide has altered biochemical properties with severe pathological consequences. The enhanced toxicity is likely due to the higher aggregation propensity and the longer bioavailability of the Aβ\textsubscript{pe3} oligomers.
gested the presence of hypothetical peptidases cleaving the first two amino acids. On the other hand, \( \text{A}\beta_{1-40} \) starting with a glutamate residue is known to be generated by alternative BACE1 cleavage of APP in the trans-Golgi network (43) and is the precursor for \( \text{A}\beta_{\text{pE}1-40} \). After exposure of the free glutamate the enzyme glutaminyl cyclase (QC) catalyzes pyroglutamate formation by dehydration of glutamate (44).

**Biochemical Properties of Pyroglutamate \( \text{A}\beta \)**

The conversion of \( \text{A}\beta \) into \( \text{A}\beta_{\text{pE}3} \) leads to altered biophysical and biochemical characteristics pointing to changes in aggregation and stability. Saido et al. (45) suggested that \( \text{A}\beta_{\text{pE}3} \) and other modified \( \text{A}\beta \) species accumulate because of their limited degradation. The formation of the lactam ring and the loss of two negative charges and one positive charge result in higher hydrophobicity and thus more stability and aggregation propensity of the \( \text{A}\beta_{\text{pE}3} \) peptides (45). In addition, the formation of the N-terminal pyroglutamate, which is resistant to degradation by peptidases, increases the stability of the peptide. He and Barrow (46) reported that \( \text{A}\beta_{\text{pE}3} \) peptides show enhanced \( \beta \)-sheet formation and aggregation propensity in aqueous and hydrophobic media compared with full-length \( \text{A}\beta \). They suggested that the loss of three charged groups facilitates and stabilizes \( \beta \)-sheet formation by reducing the level of unfavorable charge repulsion between strands (46). Using a mixture of \( \text{A}\beta \) species including \( \text{A}\beta_{\text{pE}3} \) levels mimicking the amount present in AD patients, abundant globular structures and higher aggregation rates were observed. In contrast, the aggregation propensity using the mixture present in non-demented individuals was significantly lower (47). Interestingly, \( \text{A}\beta_{\text{pE}3} \) displayed up to 250-fold accelerated formation of aggregates compared with \( \text{A}\beta_{1-40} \) irrespective of the C terminus of \( \text{A}\beta \) (48).

Russo et al. (49) claimed that \( \text{A}\beta_{\text{pE}3-40} \) is more toxic for neurons and astrocytes compared with full-length \( \text{A}\beta_{1-40} \). Similarly, Piccini et al. (47) showed that an \( \text{A}\beta \) mixture similar to those found in brains of AD patients increased cell membrane permeability, leading to reduced survival of neuroblastoma cells.

It is worth mentioning that in contrast to what has been described above, some studies have indicated that the second structure and toxicity of \( \text{A}\beta_{\text{pE}3-40/42} \) peptides are similar to those of \( \text{A}\beta_{1-40/42} \) peptides (50, 51). In agreement, Youssef et al. (52) showed that \( \text{A}\beta_{1-42} \) and \( \text{A}\beta_{\text{pE}3-42} \) exhibited similar effects on neuronal cytotoxicity in primary cortical neurons and on memory impairment after intracerebroventricular injection in wild-type mice.

**Pyroglutamate \( \text{A}\beta \) in Transgenic Alzheimer Mouse Models**

The development of transgenic mouse models had a remarkable influence on our understanding of the pathological alterations underlying AD. The predominance of the \( \text{A}\beta_{\text{pE}3} \) Peptide in AD and DS patients and its characteristics raised the question of different \( \text{A}\beta \) isoforms in AD mouse models.

In the commonly used Tg2576 mouse model, truncated and modified \( \text{A}\beta \) appears at 16 months of age and comprises only 5% of the total \( \text{A}\beta \) peptides even at an old age of 23 months (53). The scarcity of modified \( \text{A}\beta \) was suggested as one of the reasons for the lack of neurofibrillary pathology and neuronal loss in Tg2576 brain. \( \text{A}\beta_{\text{pE}3} \) and other modified forms of \( \text{A}\beta \) are absent in another mouse model, APP23, until 22 months of age. The difference in the composition of \( \text{A}\beta \) pools between AD and the APP23 mouse model might account for the different plaque morphology and the high solubility of \( \text{A}\beta \) peptides in APP23 mice (54). The discrepancy in the \( \text{A}\beta \) isoforms between AD and mouse models might be due to the short life span of the mice compared with the decades available for \( \text{A}\beta \) post-translational modification in AD.

Interestingly, there are other AD mouse models available that produce high levels of \( \text{A}\beta_{\text{pE}3} \). They develop early neurological deficits and more severe pathology. Most of these models harbor more than one mutation to trigger AD pathology. The APP/PS1KI mouse model is a double transgenic model with four mutations in APP and presenilin-1 (PS1) (55). It develops age-dependent plaque deposition and intracellular \( \text{A}\beta \) accumulation including \( \text{A}\beta_{\text{pE}3} \) as early as 2 month of age (56). The area covered with \( \text{A}\beta_{\text{pE}3} \)-positive plaques increases from <1% at 2 months of age to ~2% at 6 months until it covers >4.5% of the cortex at 10 months of age (41). APP/PS1KI mice exhibit robust learning deficits at 6 months (57), as well as an age-dependent axonopathy (58). In addition, significant neuron loss in the CA1 layer of the hippocampus at 6 months coincides with synaptic deficits, hippocampus atrophy, and a massive increase in intraneuronal and extracellular aggregation of \( \text{A}\beta_{\text{pE}3} \). However, neuron loss seen in the CA1 region correlates with intraneuronal rather than extracellular \( \text{A}\beta \) (59).

The 5xFAD mouse model is another double transgenic APP/PS1 mouse line coexpressing five familial AD mutations that are inherited together, leading to accelerated plaque formation and increased \( \text{A}\beta_{1-42} \) levels (60). It recapitulates a variety of AD hallmarks that appear at an early age, including working memory impairment, altered anxiety corresponding to disinhibitory tendencies seen in AD patients, and motor impairments. In addition, it shows extensive extracellular plaque formation and selective neuron loss in the fifth cortical layer that correlates with intraneuronal \( \text{A}\beta \) accumulation in this layer (61).

Despite the value of the APP/PS1KI and 5xFAD mouse models in providing \( \text{A}\beta \) heterogeneity, which is similar to that seen in AD, this heterogeneity makes it also difficult to distinguish the individual toxic peptides from those that might only coprecipitate and could be potentially less harmful. Therefore, it was necessary to generate a mouse model that exclusively develops N-terminally truncated \( \text{A}\beta \). The TBA2 mouse model expresses \( \text{A}\beta_{Q3-42} \) starting with an N-terminal glutamine (Q) residue at position 3 of \( \text{A}\beta \), which facilitates the conversion to \( \text{A}\beta_{\text{pE}3} \). Glutamine was used instead of the naturally occurring glutamate because it represents a better substrate for pyroglutamate conversion (either spontaneously or enzymatically catalyzed). However, the degree of conversion was not determined. Therefore, unmodified N-terminally truncated \( \text{A}\beta \) could also contribute to the observed pathology. The mice exhibited abundant intraneuronal \( \text{A}\beta_{\text{pE}3} \) predominantly in the hippocampus and Purkinje cells of the cerebellum, which was associated with loss of Purkinje cells, cerebellar atrophy, striking neurological impairment, and growth retardation. TBA2 mice revealed a
>50-fold increased $\text{A}\beta_{\text{pE3–42}/\text{A}\beta_{\text{A–42}}}$ ratio compared with 6-month-old APP/PS1K1 mice (62).

**Soluble Oligomeric Pyroglutamate $\text{A}\beta$: The Missing Link in $\text{A}\beta$ Toxicity?**

For more than 2 decades, the amyloid hypothesis has been the central hypothesis in coining the molecular pathology of AD (63). This hypothesis argued that amyloid fibrils, which are large insoluble polymers of $\text{A}\beta$ found in senile plaques, are the trigger of neuron loss and dementia typical for AD. Albeit the convincing genetic, biochemical, and cell biological data for a major role of $\text{A}\beta$ in AD, growing evidence points toward soluble $\text{A}\beta$ oligomers.

One of the major flaws in the amyloid hypothesis is the weak correlation between the severity of dementia and the density and localization of amyloid plaques in the brains of AD patients. Memory impairment and pathological changes in many AD mouse models occur before the first signs of plaque deposition (64). Soluble oligomers are low molecular weight non-fibrillar structures, which are stable in aqueous solution and remain soluble even after high speed centrifugation (64). $\text{A}\beta$ oligomers preferentially develop intracellularly within neuronal processes and synapses rather than within the extracellular space (65, 66). Results from several laboratories suggest these oligomers to be the missing link in the amyloid hypothesis. Although $\text{A}\beta$ plaques are poor correlates for the clinical symptomatology in AD and DS patients, soluble oligomers are suggested to be good predictors for synaptic loss (67), neurofibrillary tangles (68), and clinical phenotype (69, 70). Just as in the human brain, studies using AD mouse models support the role of oligomers. In the Tg2576 mouse model, the appearance of $\text{A}\beta$ dodecamers coincided with the onset of spatial memory impairment. Interestingly, injection of these purified oligomers into the ventricles of wild-type rats caused a dramatic drop in spatial memory performance (71). With regard to short-term effects, oligomers have been shown to impair synaptic plasticity by blocking long-term potentiation and reinforcing long-term depression (72). Tomiyama et al. (73) generated APP transgenic mice expressing the E693A mutation, which causes neuronal cell death and cognitive impairment by enhanced intracellular $\text{A}\beta$ oligomerization without plaque formation.

With the abovementioned considerations, oligomers are thought to be a good target for therapeutic antibodies especially since this pool represents a minor subset (~1.4% of the total $\text{A}\beta$) compared with the plaque pool, which occupies the whole parenchyma (68). Reports have shown that monoclonal antibodies raised against oligomers prevent oligomer-induced toxicity, production of reactive oxygen species, and their attachment to synapses in primary hippocampal cells (74). In addition, Klyubin et al. (75) have shown that immunization against $\text{A}\beta$ oligomers neutralized long-term potentiation inhibition induced by $\text{A}\beta$ oligomers injected in rat hippocampus. Analysis of water-soluble $\text{A}\beta$ in AD, DS, and non-demented elderly brain specimens indicated the presence of $\text{A}\beta_{1–42}$, $\text{A}\beta_{\text{pE3–42}}$, and $\text{A}\beta_{\text{pE11–42}}$. In DS, water-soluble $\text{A}\beta$ appeared early (~20 years before the appearance of the plaques) and increased with age and the progression of the amyloid pathology. Interestingly, water-soluble $\text{A}\beta$ increased by 100-fold in young cases accompanied by an increase in $\text{A}\beta_{\text{pE3–42}}$ (76). Russo et al. (77) showed that cases with a PS1 mutation developed a higher ratio of water-soluble $\text{A}\beta_{\text{pE3–42}}$ and $\text{A}\beta_{\text{pE11–42}}$ to full-length $\text{A}\beta_{1–42}$ in comparison with sporadic AD cases. In line with this observation, water-soluble $\text{A}\beta$ from brains of normal elderly individuals with abundant amyloid and neurofibrillary pathology demonstrated a decreased $\text{A}\beta_{\text{pE3–42}}/\text{A}\beta_{1–42}$ ratio compared with AD cases (76). Overall, the ratio of water-soluble $\text{A}\beta_{\text{pE3–42}}$ to $\text{A}\beta_{1–42}$ seems to be proportional to the clinical phenotype and the severity of the disease.

**Passive Immunization against Low Molecular Weight Pyroglutamate $\text{A}\beta$ Oligomers**

A recently generated novel monoclonal antibody (9D5) was used to demonstrate that it is possible to detect low molecular weight pyroglutamate-modified $\text{A}\beta$ oligomers (51). The selectivity of the antibody for low molecular weight (4–10-mers) pyroglutamate-modified $\text{A}\beta$ was confirmed by size exclusion chromatography and immunoblot assays. When the 9D5 antibody was added to $\text{A}\beta_{\text{pE3–42}}$ monomers, it efficiently decreased the formation of higher aggregates of the $\text{A}\beta_{\text{pE3–42}}$ peptide but did not interfere with the rapid formation of $\text{A}\beta_{1–42}$ aggregates. Furthermore, adding the 9D5 antibody to SY5Y neuroblastoma cells completely abolished the toxic effects of $\text{A}\beta_{\text{pE3–42}}$ peptides, whereas the toxicity of $\text{A}\beta_{1–42}$ was unaltered. Interestingly, the 9D5 antibody showed a specific staining pattern in AD cases, differentiating between non-demented control cases and AD. Passive immunization of 4.5-month-old 5XFAD mice with the 9D5 antibody for 6 weeks was capable of reducing overall $\text{A}\beta$ plaque load and $\text{A}\beta_{\text{pE3–x}}$ levels, leading to a normalization of the behavioral phenotype. Based on that, 9D5 represents a therapeutically and diagnostically effective monoclonal antibody targeting low molecular weight $\text{A}\beta_{\text{pE3}}$ oligomers (51).

Several mechanisms were proposed as potential modes of action for the clearance of $\text{A}\beta$ via immunotherapy. The first mechanism is that antibodies act catalytically to dissolve preformed $\text{A}\beta$ aggregates or prevent $\text{A}\beta$ aggregation (78). A second mechanism relies on binding of the antibodies to $\text{A}\beta$, followed by interaction of the Fc part of the antibody with the Fc receptor on the microglial surface, leading to increased $\text{A}\beta$ phagocytosis (79). These mechanisms assume that sufficient antibodies cross the blood-brain barrier and bind to the amyloid. Another option does not require the penetration of the blood-brain barrier. The peripheral sink hypothesis was demonstrated for the first time with the $\text{A}\beta$ antibody m266 and suggests that the presence of circulating antibodies in plasma alters the equilibrium and favors the efflux of $\text{A}\beta$ toward the periphery due to an $\text{A}\beta$ concentration gradient (80). It was demonstrated that the central domain $\text{A}\beta$ monoclonal antibody m266 can act as an $\text{A}\beta$ sink both in vitro and in vivo and that parenteral administration of it can alter the equilibrium of $\text{A}\beta$ between the central and peripheral compartments. Another mechanism proposes the ability of certain antibodies to bind to oligomers and neutralize their synaptotoxic effects directly (75). It is worth mentioning, however, that these mechanisms are not exclusive and may overlap under different circumstances. Moreover, different states of the disease might require one mechanism more than the other (81).
Based on that, antibodies that are exclusively selective for oligomeric Aβ are promising tools for therapeutic intervention in AD for many reasons. As mentioned above, Aβ oligomers represent <2% of the total Aβ pool in the brain, which makes them an achievable therapeutic target especially assuming that only a minor amount of the antibodies can cross the blood-brain barrier (0.11% of the circulating antibodies enter the brain (82)). Thus, targeting oligomeric Aβ can tremendously reduce the amount of antibodies needed to achieve the desired effect in comparison with fibrillar (plaque) Aβ, representing the predominant species in AD brain. Also, antibodies against Aβ oligomers bind the pernicious toxic species and decrease the building units for fibrils, thereby hindering plaque formation. On the other hand, immunization against fibrillar forms may dissolve the plaques into soluble Aβ forms and thus increase the potentially toxic species in the brain. Furthermore, immunization against the oligomeric Aβ minute species might spare patients from the drawbacks of some clinical trials such as microbleeds and hemorrhages that might result from an excessive immune reaction against Aβ plaques (83).

Pyroglutamate Aβ Cyclization Is Catalyzed by Glutaminyl Cyclase

QC belongs to the metal-dependent acyltransferase family, converting glutamine (or alternatively glutamate) into pyroglutamate with the liberation of ammonia (or water) (84). In vitro and in vivo experiments indicated the presence of QC in the secretory pathway (endoplasmic reticulum, Golgi apparatus, and secretory granules) (85–87).

A recent study has shown that complete genetic depletion of QC in mice resulted in a 24% decrease in thyroxine, suggesting mild hypothyroidism. However, QC knock-out (KO) mice showed similar blood glucose and glucose tolerance levels compared with wild-type mice. Other parameters related to QC activity, such as serum gonadotropin-releasing hormone, thyroid-stimulating hormone, and testosterone concentrations, were not changed by QC depletion (88).

Compelling evidence demonstrates the role of QC in the generation of AβpE. Incubation of synthetic Aβ1–x with recombinant QC resulted in conversion to AβpE1–x, a reaction that is favored under acidic pH conditions and blocked by the presence of a QC inhibitor (44). Furthermore, in two different cell lines, QC stimulated AβpE1 generation (89, 90). Application of a QC inhibitor suppressed the cyclization reaction to AβpE1. Interestingly, addition of recombinant QC to culture media generated only minor amounts of AβpE1. This finding indicates that pyroglutamate Aβ formation is favored within intracellular compartments (89).

In agreement with in vitro studies, several in vivo reports have supported the role of QC in the production of AβpE. Injection of Aβ1–40 into wild-type rats led to significant production of AβpE1–40 within 24 h, which was inhibited by co-application of a QC inhibitor (91). Oral administration of a QC inhibitor to Tg2576 and Tg2576 transgenic mice reduced AβpE1, Aβ40, and Aβ42 levels. This was accompanied by a reduction in plaque load and gliosis in addition to improvements in contextual fear memory and spatial memory. Simi-
larity, treatment of transgenic *Drosophila* expressing Aβ<sub>P3–42</sub> for 4 weeks with a QC inhibitor led to reduced Aβ<sub>P3–42</sub> levels (92).

The use of transgenic or knock-out mice strongly facilitates analysis of the role of gene function *in vivo*. To study the effect of ectopic human QC (hQC) overexpression, 5xFAD mice were crossed with transgenic mice expressing hQC under the control of the neuron-specific Thy1 promoter. 5xFAD/hQC bigenic mice showed significantly increased levels of TBS-, SDS-, and formic acid-soluble Aβ<sub>P3–42</sub> peptides and aggregation in plaques. 6-month-old 5xFAD/hQC mice developed significant motor and working memory impairment. The effect of endogenous QC was studied by generating 5xFAD/QC-KO mice (homozygous for murine QC knock-out). 5xFAD/QC-KO mice showed a significant reduction in Aβ<sub>P3–42</sub> levels, decreased plaque pathology, and a rescue of the behavioral phenotype (93). These data clearly demonstrate that QC is a key participant in modulating Aβ<sub>P3–42</sub> formation. Fig. 2 summarizes the potential therapeutic actions to inhibit and/or clear Aβ<sub>P3–42</sub> oligomers from AD brain.

**Pyroglutamate as a Potential Diagnostic Marker**

The diagnosis of AD relies on neuropsychological tests, neuroimaging, and cerebrospinal fluid biomarkers. Nonetheless, the exact diagnosis is not definite unless the autopsied brain is examined and neuropathologically evaluated. Pittsburgh consortium criteria (94) demonstrated that the [11C]PIB signal correlated with the localization and abundance of Aβ<sub>P3–42</sub>-positive plaques. An *in vitro* binding assay revealed that specific binding of [11C]PIB to Aβ<sub>P3–42</sub> fibrils was 4–5-fold higher than to Aβ<sub>P3–42</sub> fibrils. Britschgi *et al.* (95) observed that many AD and healthy control plasma samples showed high levels of IgG autoantibodies against Aβ<sub>P3–42</sub> and Aβ<sub>P11–42</sub>. In addition, the titer of IgM autoantibodies against Aβ<sub>P3–42</sub> correlated with the cognitive status of individuals at risk to develop AD (96). In good agreement, the level of Aβ<sub>P3–42</sub> oligomers was significantly decreased in the plasma of AD patients (51). However, it is noteworthy that these studies are pilot studies with small group sizes and need to be further replicated and confirmed using larger cohorts of patients and controls.

In summary, compelling evidence of a significant contribution Aβ<sub>P3–42</sub> has been accumulated since its discovery in 1992. Its specific biochemical properties and the molecular events controlling the formation of Aβ<sub>P3–42</sub> provide a better understanding of the pathology leading to AD, and it has the potential as a target for therapy as well as a marker for diagnosis. Although Aβ<sub>1–42</sub> is a toxic peptide, a normal physiological function cannot be excluded. Current knowledge indicates that Aβ<sub>P3–42</sub> is a solely pathological cousin of full-length Aβ acting as a "hatchet man" in AD.

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