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Aminopeptidase A and dipeptidyl peptidase 4: a pathogenic duo in Alzheimer's disease?

Frédéric Checler*, Audrey Valverde

The etiology of Alzheimer's disease is far from being completely understood. Genetic approaches have helped in this matter and have greatly supported the view that the β-amyloid precursor protein (βAPP) could be at the center of gravity of the pathology. Thus, mutations responsible for autosomal dominant aggressive forms of Alzheimer's disease (AD) are all harbored by either βAPP itself or by its cleaving enzyme presenilins 1/2 referred to as γ-secretase. It was therefore convincing to note that fully independent gene products harboring AD-linked mutations, all concur to modulate βAPP proteolytic processing. These genetic clues combined with a bulk of anatomical observations and cellular manipulations pointed to the role of amyloid-β (Aβ), the main biochemical component of senile plaques that accumulate at late stages of AD. Unfortunately, a series of clinical trials designed to either abolish Aβ production or neutralize it once produced have consistently failed (Checler et al., 2021). It remains that the genetic arguments are strong and that a key role of βAPP proteolytic maturation remains of actuality. One way to reconcile genetic evidence and clinical trials failure could be to envision that additional βAPP-derived products could contribute to AD etiology. A close evaluation of biogenesis and toxicity of such pathogenic βAPP-derived products, distinct from genuine Aβ could help to better understand AD etiology.

One potential etiological trigger of AD could be the β-secretase-derived APP-C-terminal fragment, namely C99. This fragment triggers cellular dysfunctions reminiscent of those observed in AD-affected brains such as endolysosomal and autophagic alterations, mitochondrial structure and function defects as well as apathy-like behavior (Lauritzen et al., 2016; Bourgeois et al., 2018). Of importance, it was reported that these cellular perturbations were independent of Aβ and were even worsened by γ-secretase inhibitors (Checler et al., 2021), and that this product accumulated in AD brains (Pulina et al., 2020).

An additional secretase, referred to as η-secretase recently came on stage. η-secretase, belongs to the family of disintegrins and has been identified as MT5-MMP. It gives rise to a η-CTF fragment that undergoes subsequent cleavages by β- and α-secretases, thereby yielding Aηβ and Aηα, respectively. Although MT5-MMP deficiency reduces amyloid and C99 burdens and alleviates neuroinflammation and cognitive deficits (Baranger et al., 2016), the definitive implication of MT5-MMP in AD pathology remains to be established.

Aβ peptide also undergoes secondary cleavages at both N- and C-terminal ends (Dunys et al., 2018). This can be seen as a degradation process aimed at clearing off an excess amount of Aβ or alternatively, can be seen as a biotransformation process yielding shorter peptides harboring their own function or toxicity. The pyroglutamate 3-Aβ peptide (pE3-Aβ) has attracted recent attention. First, pE3-Aβ accumulates early not only in transgenic mice brains but also in AD and Down syndrome-affected brains (Bayer, 2021). Second, the enzyme triggering the cyclization of the glutamate residue in position 3 has been identified as glutaminyl cyclase (QC). A bulk of evidences underlines the putative importance of QC in AD. Among them, both genetic depletion and pharmacological blockade of QC lower pE3-Aβ load and improve AD-related memory deficits (Schilling et al., 2008).

The genesis of pE3-Aβ is a two steps process that theoretically requires first exopeptidasic removal of N-terminal aspartyl and then alanine residues to yield E3-Aβ that undergoes subsequent cyclization by QC (**Figure 1**). A simple observation of the nature of the N-terminal aspartyl residue of Aβ led us to envision the participation of an enzyme avid for acidic residues. Theoretically, aminopeptidase A (APA) that displays a high affinity for such structural features appeared as a putative candidate. Our initial evidence that APA could well contribute to the first catalytic step ultimately yielding E3-Aβ was deduced from a pharmacological approach. Thus, we showed that the APA inhibitor (RB150) was able to enhance the recovery of intact full-length Aβ produced by various cell lines (Sevalle et al., 2009). This data encouraged us to strengthen our data by examining APA contribution in Aβ truncation by combined biochemical, cellular, *ex vivo* and *in vivo* approaches (Valverde et al., 2021b).

First, we showed by mass spectrometry that human recombinant APA indeed liberated the aspartyl 1 residue of synthetic Aβ. Second, we demonstrated that the pharmacological blockade of APA by selective APA inhibitors restored the normal distribution of mature spines and reduced the proportion of filipodia in Swedish-mutated APP-infected organotypic hippocampal slices prepared from young mice. Of interest, by both pharmacological treatment and genetic targeting of endogenous APA by shRNA approach, we observed that APA blockade/reduction not only drastically reduced loads of soluble and insoluble pE3-$A\beta_{42}$ but also lowered $A\beta_{42}$ expression and the number (but not mean perimeter and area) of $A\beta_{42}$-positive plaques of 12-month-old *3xTg-AD* mice. The fact that the APA inhibitor reduced pE3-Aβ and Aβ42 indicated a close relationship between the two peptides that could be supported by a physical interaction. Thus, it had been shown that pE3-Aβ could drastically increase Aβ propensity to aggregate and could serve as a seed of Aβ, thereby exacerbating its deposition. This observation could be of therapeutic value. Thus, the recent development of a monoclonal antibody referred to as aducanumab, that selectively targets Aβ aggregates (Sevigny et al., 2016), proved useful to significantly reduce both soluble and insoluble Aβ. Further, although it remains highly controversial, it was shown that monthly intravenous administration of aducanumab at early stages of AD apparently slowed down cognitive decline. When comparing negative clinical outcomes of Aβ-centric immunotherapy, it indicates that as far as one stick to the amyloid cascade hypothesis, targeting aggregates versus monomeric Aβ could be of better value. In this context, our observation that APA inhibitor could circumvent Aβ aggregation and plaques formation by protecting Aβ from its N-terminal truncation completely validates, at least to some extent, this possible therapeutic track. It is of note that one of the prevalent truncated Aβ forms is the $A\beta_{4-40/42}$, meaning that E3-Aβ could potentially escape GC-mediated cyclization and be further processed (**Figure 1**) (Bayer, 2021). As the third N-terminal residue of Aβ is glutamate, it is not unrealistic to

speculate on the involvement of APA in the release of this acidic amino acid, but this remains to be firmly established.

Biochemical and anatomical data supporting the putative contribution of APA to the AD-like pathogenic process were strengthened by behavioral results (Valverde et al., 2021b). We showed that both pharmacological blockade and genetic reduction of APA ameliorated the learning and memory of 3×Tg-AD. Thus, shRNA directed towards APA improved the primary escape latency and augments the number of entries in every hole in the Barnes maze assay. In the water maze, APA reduction lowers the latency to reach the platform and augments the number of entries in the target quadrant. These observations were fully reproduced by treatment with the specific APA inhibitor RB150. Overall, our biochemical, *in situ* and *in vivo* data all concur to identify APA as a key contributor to Aβ truncation and associated AD-like pathology. However, our behavioral studies also indicated that APA reduction did not fully rescue wild-type phenotypes (Valverde et al., 2021b). Several possibilities stand to explain the latter observation. First, one could envision that the APA inhibitor only partially inhibits its target due to limited accessibility/bioavailability leading to *in situ* concentrations below the saturating concentrations needed to completely block APA. The same could stand for the ability of shRNA to completely inactivate endogenous APA. An alternative explanation could be that APA only partly contributes to Aβ truncation and that an additional enzymatic activity could participate in Aβ exopeptidasic degradation.

The latter hypothesis appeared likely and we envisioned the possibility that, besides the sequential liberation of aspartyl and alanyl residues, the direct release of the N-terminal Asp-Ala dipeptide of Aβ (**Figure 1**) could well occur. Based on its structural preference for natural substrates, dipeptidyl peptidase 4 (DPP4) fulfilled this criterion. Thus, DPP4 greedily releases X-Ala dipeptides and accordingly, one of its natural substrates is Glucagon-like peptide 1 (GLP-1), which harbors a His-Ala sequence at its N-terminal moiety. Of note, GLP-1 is present in the hippocampus and frontal cortex and is decreased in AD mouse brain and AD-affected patients, thus underlining a putative pathogenic contribution of DPP4. We thus examined the possibility that DPP4 could, besides APA, contribute to pE3-Aβ production.

The first clue that this hypothesis could be valid came from cell biology approach

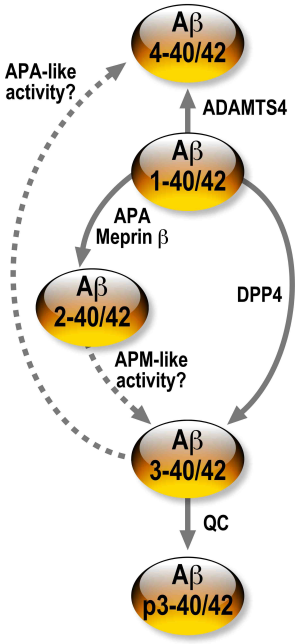

Figure 1 | Amyloid β (Aβ) truncation: products and enzymes.
Amyloid $\beta_{1-40/42}$ can be cleaved by aminopeptidase A (APA) that releases $A\beta_{2-40/42}$ or by dipeptidyl peptidase 4 that liberates the N-terminal dipeptide, thereby yielding $A\beta_{3-40/42}$. $A\beta_{3-40/42}$ can be cyclized by glutaminyl cyclase. Dashed lines indicate putative cleavages by aminopeptidase M (APM) and APA. An additional N-terminal truncation triggered by ADAMTS4 can directly produce $A\beta_{4-40/42}$. DPP4: Dipeptidyl peptidase 4.

where we treated cells overexpressing Swedish-mutated APP with the selective DPP4 inhibitor sitagliptin (Valverde et al., 2021a). We observed a clear increase of full-length Aβ recovery suggesting protection towards exopeptidasic proteolysis. However, interestingly, we demonstrated that sitagliptin- and RB150-mediated effects were additive, suggesting that both APA and DPP4 could act in concert to truncate Aβ. Subsequent physicochemical, anatomical and behavioral studies confirmed this view (Valverde et al., 2021a). Firstly, mass-spectrometry analysis indicated that recombinant DPP4 hydrolyzed synthetic Aβ directly at the Ala2-Glu3 peptidyl bond, thereby releasing E3-Aβ; secondly, sitagliptin restored wild-type-like synaptic morphology in hippocampal organotypic slices; Thirdly, both sitagliptin and DPP4-directed shRNA drastically lowered $A\beta_{42}$-positive plaques and as well as $A\beta_{40}$ and $A\beta_{42}$ loads in 3xTg-AD mice brains (Valverde et al., 2021a); Fourth, both DPP4 shRNA and sitagliptin partly rescued AD-like defects observed in learning and memory tasks in 11–12-month-old 3×Tg-AD mice (Valverde et al., 2021a). This set of data agreed well with previous works documenting a protective role of DPP4 inhibitors in cognitive disorders. This was mainly explained by the blockade of DPP4-mediated cleavage of GLP-1 of DPP4 in a type 2 diabetes context, which is a well-recognized risk factor condition for the deterioration of cognitive function linked to dementia syndrome.

Overall, our data support the possibility that APA and DPP4 could efficiently participate to the Aβ N-terminal truncation. According to such postulate, we should expect augmentation of their expressions and/or activities in AD-affected brains. If one agrees with the observation that pE3-Aβ occurs after Aβ production but later contributes to its accumulation and seeding, one should expect an increase of APA and DPP4 activities at the early stages of the pathologic process. In this context, we took advantage of a rather large cohort of control and sporadic AD brains that were categorized according to Braak and Thal stages and assessed for amyloid angiopathy (Valverde et al., 2021a, b). Of most interest, we observed a transient and early increase of APA activity at Braak stages I–III (Valverde et al., 2021a, b). The same transient augmentation in DPP4 activity was observed. This set of data not only indicated that both APA and DPP4 could contribute to pE3-Aβ production but that their activity was transiently and concomitantly enhanced in AD-affected brains.

Could we envision a therapeutic option to interfere with either the pE3-Aβ load or APA and DPP4 activities? pE3-Aβ-directed monoclonal antibody referred to as donanemab was recently designed and used in clinical trials. This immunological probe proved useful to drastically reduce the number of plaques by about 80% in patients presenting a debuting or mild AD pathology. This was accompanied by a slow-down of the decline of one out of five cognitive measurements. However, a deleterious effect was observed on brain volume observed by magnetic resonance imaging (Ayton, 2021). Besides such immunotherapy, one can envision a direct but risky and hazardous genes therapy targeting APA and DPP4. However, although one can not preclude theoretical side effects due to the protection of various endogenous substrates of these enzymes by their selective inhibitors, we believe that the most likely possibility remains to pharmacologically block these two enzymes. The advantage of such a strategy stands in the fact that both APA and DPP4 inhibitors have been either already used in clinics or successfully passed the phase II clinical assessment. Thus, DPP4 inhibitors were used for the treatment of GLP-1-linked type 2 diabetes

(Gallwitz, 2019) while orally available APA inhibitors are seen as a potential treatment for central hypertension and heart failure (Marc et al., 2020). Therefore, their use in AD cases would be rendered possible without the tedious need for preclinical investigations and the benefit/risk ratio for AD treatment appears high.

Our data indicate that inhibitors or APA or DPP4 alone only partly protect against AD-like stigmata and cognitive defects. In this context, it is reasonable to consider that a "monotherapy" would not prove sufficiently efficient. One should better envision a combination of the two types of inhibitors to trigger a beneficial effect, in an AD context. This statement raises several problems that need to be addressed if one envisages that both inhibitors must act concomitantly at the adequate site, with similar affinity/potency and should display similar pharmacokinetics. Thus, the co-administration of the two separate inhibitors would be likely difficult to achieve. An alternative would be to generate compounds where APA and DPP4 inhibitors are tied by a chemical linker. These "mixed" inhibitors should prove useful to release equimolar amounts of both inhibitors and at similar cerebral sites to target Aβ. The current approach is in progress in our laboratory to develop such compounds.

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Frédéric Checler*, Audrey Valverde
Université Côte d'Azur, INSERM, CNRS, IPMC, team labelled "Laboratory of Excellence (LABEX) DistAlz", Valbonne, France

***Correspondence to:** Frédéric Checler, PhD, checler@ipmc.cnrs.fr.
https://orcid.org/0000-0003-2098-1750 (Frédéric Checler)

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