

# Is $\gamma$ -secretase a beneficial inactivating enzyme of the toxic APP C-terminal fragment C99?

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Genetic, biochemical, and anatomical grounds led to the proposal of the amyloid cascade hypothesis centered on the accumulation of amyloid beta peptides ( $A\beta$ ) to explain Alzheimer's disease (AD) etiology. In this context, a bulk of efforts have aimed at developing therapeutic strategies seeking to reduce  $A\beta$  levels, either by blocking its production ( $\gamma$ - and  $\beta$ -secretase inhibitors) or by neutralizing it once formed ( $A\beta$ -directed immunotherapies). However, so far the vast majority of, if not all, clinical trials based on these strategies have failed, since they have not been able to restore cognitive function in AD patients, and even in many cases, they have worsened the clinical picture. We here propose that AD could be more complex than a simple  $A\beta$ -linked pathology and discuss the possibility that a way to reconcile undoubted genetic evidences linking processing of APP to AD and a consistent failure of  $A\beta$ -based clinical trials could be to envision the pathological contribution of the direct precursor of  $A\beta$ , the  $\beta$ -secretase-derived C-terminal fragment of APP,  $\beta$ CTF, also referred to as C99. In this review, we summarize scientific evidences pointing to C99 as an early contributor to AD and postulate that  $\gamma$ -secretase should be considered as not only an  $A\beta$ -generating protease, but also a beneficial C99-inactivating enzyme. In that sense, we discuss the limitations of molecules targeting  $\gamma$ -secretase and propose alternative strategies seeking to reduce C99 levels by other means and notably by enhancing its lysosomal degradation.

Alzheimer's disease (AD) is the most frequent age-related neurodegenerative disease. After initial clinical characterization, histopathological analysis revealed the presence of two major anatomical lesions signing this pathology: senile plaques that are extracellular protein aggregates and neurofibrillary tangles that are intracellular neuronal lesions (1–3). Senile plaques are mainly composed of the small 4 kDa amyloid beta peptide ( $A\beta$ ) (4) and neurofibrillary tangles of hyperphosphorylated and cleaved forms of the microtubule-associated protein tau (5, 6). The cloning of the  $A\beta$  precursor, the  $\beta$ -amyloid precursor protein (APP), in the late 80s was a key step in the understanding of the pathology. APP was found to be localized on chromosome 21 (7), thus explaining the

development of early-onset dementia and the presence of senile plaques in Down syndrome patients carrying an extra copy of APP due to a duplication of this chromosome (8). Furthermore, the identification of APP mutations responsible for autosomal dominant form of AD (FAD), including the "Dutch" (9) "London" (10) and "Swedish" mutations (11), showed that the functional consequences of these mutations were to augment the load of  $A\beta$  and/or to shift  $A\beta$  production to more aggregating  $A\beta$  peptides (12, 13). The role of  $A\beta$  in AD etiology was further confirmed by the discovery few years later of the first mutations in presenilins that were found to be involved in  $A\beta$  production and, similarly to APP mutations, seemed to exacerbate  $A\beta$  accumulation in cells, animal models as well as in AD-affected human brains (14–16). A last, but very important genetic evidence of a key role of  $A\beta$  in AD etiology was the recent findings of the Icelandic APP mutation that was shown to be protective by reducing cognitive decline and  $A\beta$  load by about 40% (17).

Thus, this set of histopathological, genetic, and biochemical data concurred to support the view that  $A\beta$  accumulation could be the etiological cause of the pathology, as stated in the amyloid cascade hypothesis that was proposed in 1992 by Hardy and Higgs (18). Indeed, mutations in three distinct proteins, namely APP, PS1, and PS2, are all responsible for FAD and have in common to modulate both APP processing and  $A\beta$  production. In this context, one can understand the huge efforts aimed at determining the mechanisms and enzymes involved in  $A\beta$  production and designing potent, specific, and bioavailable inhibitors of these enzymes (19, 20) or  $A\beta$  neutralizing antibodies. However, until so far, the outcomes of these  $A\beta$ -centered strategies have been extremely disappointing in our quest for meaningful treatments (21, 22) (Table 1). This has led to question the validity of the amyloid cascade hypothesis (23) or to discuss in a more cautious and balanced manner the ins, outs, and limitations of the procedures of clinical trials (24). It remains that before "throwing out the baby and the bath water," one should try to reconcile undoubted genetic evidences linking APP processing to AD and failures of  $A\beta$ -based clinical trials. In this context, a way to reconcile these observations could be to envisage the possible contribution of other APP-derived fragments distinct from  $A\beta$  itself to AD pathology. Indeed, growing evidence proposes that the direct precursor of  $A\beta$  (see below), the  $\beta$ -secretase-derived

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fragment, C99, could be an early and main contributor to AD. Thus, in this review, we address the possibility that the failure of A $\beta$ -centric clinical trials could be explained, at least partly, by their lack of effect on C99. To go further, we describe clues and evidences suggesting that  $\gamma$ -secretase should be considered as a beneficial C99-inactivating enzyme and argument against therapeutic strategies targeting this enzyme. We instead propose alternative strategies seeking to circumvent C99 accumulation, which would then have the advantage to reduce both C99 and A $\beta$  levels.

### APP processing and APP mutations

A $\beta$  is derived from APP that undergoes sequential limited proteolysis catalyzed by proteases called “secretases” (Fig. 1A) (25). In the nonamyloidogenic pathway, cleavage by  $\alpha$ -secretase and  $\gamma$ -secretase ends up with the production of three fragments: a large secreted N-terminal fragment (sAPP $\alpha$ ), a small soluble peptide p3, and the APP intracellular domain (AICD) (Fig. 1A). In the amyloidogenic pathway, a first cleavage of APP by the  $\beta$ -secretase is followed by  $\gamma$ -secretase cleavage. Again, the cleavage by  $\beta$ -secretase generates a large soluble extracellular secreted domain (sAPP $\beta$ ) and the remaining membrane stub, the  $\beta$ -secretase-derived fragment, C99, undergoes  $\gamma$ -secretase cleavage, thus liberating A $\beta$  and its C-terminal counterpart AICD (Fig. 1B). Other noncanonical cleavages on APP have also been more recently described (26). Among them, the  $\eta$ -secretase activity, carried by the matrix metalloproteinases (MT1-MMP and MT5-MMP), cleaves APP in its extracellular domain, thus producing a soluble fragment (sAPP $\eta$ ) and a membrane-bound C-terminal fragment,  $\eta$ CTF. This latter fragment can subsequently be processed by  $\alpha$ - or  $\beta$ -secretase, which will generate An $\alpha$  and C83 and An $\beta$  and C99, respectively (Fig. 1C) (27, 28).

Until so far, 28 pathogenic APP mutations have been identified with all, except the Swedish mutation, lying within the sequence of C99 ([www.alzforum.org/mutations](http://www.alzforum.org/mutations)). The Swedish variant (APP<sup>swe</sup>, KM670/671NL), although not located within C99 but two residues upstream, strongly increases the levels of this fragment, as well as those of A $\beta$  peptides, by boosting  $\beta$ -secretase cleavage (12, 13, 29). All pathogenic APP mutations lie either close to the  $\alpha$ -secretase cleavage site (the middle part of the A $\beta$  domain of C99), within the  $\gamma$ -secretase cleavage sites, or near the  $\beta$ -secretase cleavage site (N-terminal part), thus modifying the cleavages of APP by either of these secretases. While the knowledge about the exact effects of the pathogenic APP mutations is still limited, it seems that mutations close to the  $\beta$ -secretase (such as the Swedish and Leuven (E682K) mutations) favor C99 production, those close to the  $\alpha$ -secretase site (such as the Flemish [E693Q] or Arctic [E693G] mutations) lead to the generation of particular aggregation-prone A $\beta$  species, while mutations lying within the  $\gamma$ -secretase cleavage site elevate the A $\beta$ 42/A $\beta$ 40 ratio. In all cases, the development of AD pathology linked to these mutations is believed to be caused by an excessive A $\beta$  deposition (30). Nevertheless, the recent study by Xu *et al.* (31) showed that most (20 out of 28) APP mutations

not only alter A $\beta$  production, but are also less efficiently processed by  $\gamma$ -secretase and thereby also enhance the levels of C99.

### The $\beta$ -secretase

The nature of  $\beta$ -secretase was described in 1999 in four independent works and was referred to as BACE1 ( $\beta$ -site APP cleaving enzyme), memapsin 2 or ASP2 (32–35). BACE1 is an aspartic protease cleaving mainly within the Golgi and endosomes due to its optimal enzymatic activity at acidic pH (36). It cleaves APP at two distinct sites called the  $\beta$  and  $\beta'$  site, respectively. While only the cleavage on the  $\beta$ -site (Asp1) leads to A $\beta$  production (Fig. 1B), BACE1 is considered to cut APP primarily at the  $\beta'$ -site (Glu11) (Fig. 1B), thus generating the C89 fragment and a truncated nonpathological A $\beta$  peptide (A $\beta$  11-x) (36). Until so far, no mutations have been found in  $\beta$ -secretase, but the Swedish and the Leuven APP mutations are known to shift the cleavage from the  $\beta'$ -site to the  $\beta$ -site, thus leading to a much higher C99 and A $\beta$  production (12, 13, 37, 38). Inversely, the Icelandic mutation (A673T) seems to be protective against AD by decreasing  $\beta$ -secretase cleavage and thus reducing by 40% the amyloidogenic fragments sAPP $\beta$  and A $\beta$  (17, 39).

### The $\gamma$ -secretase and role of PS mutations

The first hint of the identity of the protease yielding the C-terminal end of A $\beta$ , the  $\gamma$ -secretase, was the discovery in 1995 of presenilin 1 (PS1) (40–42) and its family member presenilin 2 (PS2) (43, 44) proposed to constitute this enzyme (Fig. 2A). The definitive demonstration that presenilins could be involved in  $\gamma$ -secretase activity came from subsequent functional characterization showing that their depletion fully prevents A $\beta$  production (45, 46). However, later studies revealed that  $\gamma$ -secretase is a multiproteic complex built of not only PS1 or PS2 but also of nicastrin, the anterior pharynx defective 1 (Aph1), and presenilin enhancer 2 (PEN2) (47–49) (Fig. 2B), but the exact molecular structure of this complex was revealed by crystallography only in 2014 (50). Further studies also revealed a very complex cleavage of APP-CTFs by  $\gamma$ -secretase, since it takes place within the membrane and successively at several sites ( $\epsilon$ ,  $\zeta$ ,  $\gamma$ - and  $\gamma'$ ). The first endopeptidase cleavage ( $\epsilon$ -cleavage) can occur at two distinct sites leading to the generation of A $\beta$ 48 and A $\beta$ 49, which is then shortened by sequential carboxypeptidase cleavages (trimming) at  $\zeta$ ,  $\gamma$ - and  $\gamma'$ -sites, occurring at each three or four residues and *via* two distinct product lines (A $\beta$ 49 > A $\beta$ 46 > A $\beta$ 43 > A $\beta$ 40 > A $\beta$ 37) or (A $\beta$ 48 > A $\beta$ 45 > A $\beta$ 42 > A $\beta$ 38) and the final occurrence of peptides of varying lengths but two major species of 40 or 42 amino acid residues, respectively (51). Whereas A $\beta$ 40 is the most abundant peptide, A $\beta$ 42 is considered as the most pathogenic due to its high aggregation propensity.

So far, more than 300 mutations in presenilins are proposed to be responsible for early onset and familial forms of AD (FAD) and are now believed to comprise at least 70% of all FAD cases with the vast majority on PS1 ([www.alzforum.org/mutations](http://www.alzforum.org/mutations)). Although a huge effort has been made to try

**Table 1**  
Principal antiamyloid clinical drugs and strategies

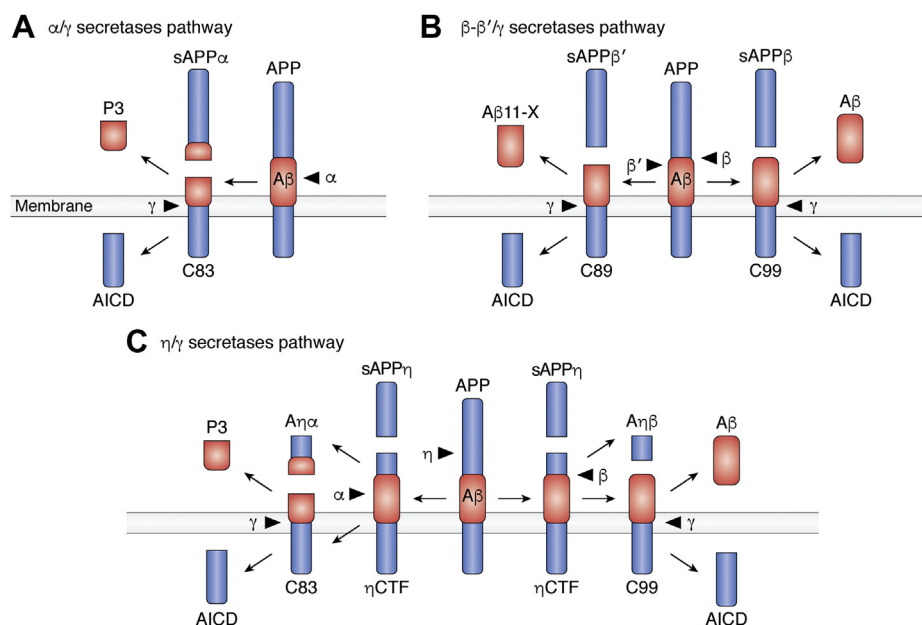
Strategy	Drug/specific target	A $\beta$ modulation in treated AD patients	FDA statute and participants	Side effects/cognitive readout	Reference
Active immunotherapy	AN-1792 (synthetic A $\beta$ 42, Janssen)	$\geq$ 60–70% A $\beta$ load reduction in the brain (post-mortem immunostaining)	Discontinued in 2002 (mild to moderate AD patients)	Meningoencephalitis	(208) (209) (210) (211)
	CAD106 (multiple copies of A $\beta$ 1-6 peptide, Novartis)	1.3% A $\beta$ reduction in brain PET scan (florbetapir) 2–3-fold increase in plasma A $\beta$ 40 at 450 mg	Discontinued in 2019 (asymptomatic carriers of APOE-4)	Worsens cognition, headache, nasopharyngitis, pyrexia, hypertension, back pain...	(212) (213)
Passive immunotherapy	Crenezumab (monomers, oligomers, and fibrils of A $\beta$ , Roche)	$\geq$ 70% A $\beta$ 42 increase in CSF at 15 mg/kg	Phase II ongoing in asymptomatic carriers of PS mutations	Lack of efficacy in mild to moderate AD	(212)
	Solanezumab (monomeric and soluble forms of A $\beta$ Eli Lilly)	170- and 18-fold increase in plasma A $\beta$ 40 and A $\beta$ 42 respectively no A $\beta$ modulation in brain PET scan (florbetapir) at 400 mg	Phase III ongoing in asymptomatic people who have biomarker evidence of brain amyloid deposition	Lack of efficacy in mild to moderate AD and in asymptomatic carriers of APP and PS mutations	(213)
	Aducanumab (oligomers, and fibrils of A $\beta$ , Biogen)	80% A $\beta$ reduction in brain PET scan (florbetapir) at 10 mg/kg	Phase III ongoing in an open-label extension study in mild to moderate AD patients	One of the two trials (EMERGE) was positive with a significant reduction in cognitive decline in mild to moderate AD a biologics license application was submitted to the FDA for approval on July 2020	<a href="http://www.alzforum.org/therapeutics/aducanumab">www.alzforum.org/therapeutics/aducanumab</a>
	Gantenerumab (oligomers, and fibrils of A $\beta$ , Roche)	$\geq$ 15%, 35% and 78% A $\beta$ reduction in brain PET scan (florbetapir) at 60, 200 and 1200 mg respectively	Phase III ongoing in an open-label extension study in mild to moderate AD patients (SCarlet RoAD, Marguerite RoAD, and GRADUATE) and in asymptomatic carriers of APP and PS mutations (Dian-Tu)	A directional trend for slower clinical decline in mild to moderate AD	(214) (215)
	BAN2401 (soluble A $\beta$ protofibrils, Biogen)	$\geq$ 120% A $\beta$ 40 increase in plasma 300 fold A $\beta$ 42 increase in CSF 93% A $\beta$ reduction in brain PET scan (florbetapir) at 10 mg/kg	Phase III ongoing in early symptomatic AD patients (Clarity AD) and in asymptomatic people who have biomarker evidence of brain amyloid deposition (AHEAD 3–45)	47% and 30% reduction in cognitive decline as judged by the ADAS-Cog and the ADCOMS respectively	(216) <a href="http://www.alzforum.org/therapeutics/ban2401">www.alzforum.org/therapeutics/ban2401</a>
$\beta$ -secretase inhibitors	Verubecestat (MK-8931Merck)	$\geq$ 57–84% A $\beta$ reduction in CSF at 12–60 mg	Discontinued in 2018 (prodromal, mild to moderate AD patients)	Worsens cognition, anxiety, depression, and sleep problems	(217) (218)
	Atabecestat (Janssen)	$\geq$ 67–90% A $\beta$ reduction in CSF at 10–50 mg	Discontinued in 2018 (asymptomatic people)	Worsens cognition, elevated liver enzymes, depression, anxiety, and sleep problems	(219) (220)
	Lanabecestat (AZD3293, Eli Lilly)	$\geq$ 64% to 78% A $\beta$ reduction in plasma at 15–50 mg $\geq$ 51% to 76% A $\beta$ reduction in CSF at 15–50 mg	Discontinued in 2018 (prodromal and mild AD patients)	Lack of efficacy on cognition, neuropsychiatric adverse events, weight loss, hair color changes	(221) (222)
	Umibecestat (Novartis)	$\geq$ 90% A $\beta$ reduction in CSF at 85 mg	Discontinued in 2019 (asymptomatic carriers of APOE-4)	Worsens cognition, brain atrophy and weight loss	(223) (187) <a href="http://www.alzforum.org/therapeutics/umibecestat">www.alzforum.org/therapeutics/umibecestat</a>
	Elenbecestat (Biogen)	$\geq$ 5.8% and $\geq$ 13.6% A $\beta$ reduction in brain PET scan (florbetaben and florbetapir respectively) at 50 mg	Discontinued in 2019 (prodromal, mild to moderate AD patients)	Weight loss, skin rashes and neuropsychiatric adverse events	(224) (225)

Table 1—Continued

Strategy	Drug/specific target	A $\beta$ modulation in treated AD patients	FDA statute and participants	Side effects/cognitive readout	Reference
$\gamma$ -Secretase inhibitors	Semagacestat (Eli Lilly)	$\geq 58.2\%$ to $64.6\%$ A $\beta$ reduction in plasma at 100–140 mg $\geq 47$ – $84\%$ newly A $\beta$ reduction in CSF at 100–280 mg $\geq 40\%$ A $\beta$ reduction in CSF at 125 mg	Discontinued in 2011 (AD patients)	<b>Worsens cognition</b> , skin cancer and infections.	(110) (111) (112)
$\gamma$ -Secretase modulators	Avagacestat (Bristol-Myers Squibb)	Not determined	Discontinued in 2012 (prodromal AD)	<b>Worsens cognition</b> , skin cancers, diarrhea, nausea, vomiting, and rash	(116)
	Rofecoxib (Merck)	No A $\beta$ 42 modulation in plasma and CSF	Discontinued in 2004 (mild to moderate AD patients)	<b>Lack of efficacy</b> , cardiovascular damage	(122)
	Tarenfluril (Myriad Genetics)	No A $\beta$ 42 modulations in CSF	Discontinued in 2009 (mild AD patients)	<b>Worsens cognition</b> , dizziness, upper respiratory tract infection and constipation.	(226) (123)
	Naproxen (Procter & Gamble)	No A $\beta$ 42 modulations in CSF	Discontinued in 2019 (asymptomatic carriers of APP and PS mutations)	<b>Lack of efficacy</b> , hypertension, gastrointestinal, and vascular or cardiac problems	(124)

ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADCOMS, Alzheimer's Disease Composite Score; CSF, cerebrospinal fluid; FDA, US Food and Drug Administration; MCI, mild cognitive impairment.

to understand the pathogenic mechanisms linked to these mutations, their exact functional consequences remain enigmatic and are probably not explained by one common mechanism. Two main, but not exclusive, hypotheses have been proposed and are still hotly debated. First, Hardy and Selkoe proposed, accordingly to the amyloid cascade hypothesis, that the effect of PS mutations should be a gain of function of  $\gamma$ -secretase (52). However, later studies revealed that most PS mutations are actually loss-of-function mutants, and a revised version of this hypothesis became focused on the relative levels of A $\beta$ 42 to A $\beta$ 40 (A $\beta$ 42/A $\beta$ 40) rather than absolute increases in A $\beta$ 42 (53). It was suggested that partial loss of PS mutations shifts the cleavage specificity of the mutant enzyme to favor A $\beta$ 42 production and that even a minor increase in A $\beta$ 42 levels could be sufficient for seed formation (54, 55). In that way, loss of function should be considered as gain of “toxic function” because of a relative increase in more aggregation-prone A $\beta$  (56). On the other hand, Shen and Kelleher proposed an alternative hypothesis “the presenilin hypothesis,” stating that loss of general PS function should be the trigger of neurodegeneration and defects in cognitive function (57). They proposed that pathogenic PS mutations behave as dominant-negative mutation due to a mechanism in which mutant PS interferes with the activity of wild-type PS, thereby reducing their physiological functions (58, 59). In agreement with this hypothesis, the extensive work of Sun and colleagues (60), in which 138 pathogenic PS mutations were analyzed, revealed that more than 90% of them led to reduced production of both A $\beta$ 40 and A $\beta$ 42, and this is in a dominant negative manner (60). This conclusion of Sun and colleagues was based on purified  $\gamma$ -secretases harboring PS mutations and assay of C99 cleavage *in vitro* or in a cell-based assay. In contrast, Szaruga and colleagues (61) measured  $\gamma$ -secretase activity in brain extracts from patients carrying PS1 mutations (one healthy and one disease allele) and found a variable effect on endopeptidase cleavage but a consistent reduction in carboxypeptidase activity, seen as A $\beta$ 38/A $\beta$ 42. These data therefore seemed to fit with the postulate that a qualitative shift in A $\beta$  profiles toward the generation of longer aggregation-prone peptides (>A $\beta$ 42) should be the common denominator of AD (61) and were in agreement with the earlier work from Saito and Saido, demonstrating a particular high toxicity of A $\beta$ 43 (62) and the initial statements of a role of aggregating-prone A $\beta$  in seed formation (63–65). However, arguing against a direct and unique role of the A $\beta$ 42/A $\beta$ 40 ratio was the findings in the work of Sun and colleagues that observed that the age of onset of FAD did not seem to correlate well with the change in this ratio (60) in contradiction to what was previously proposed (66). A recent study indicated that the pathogenicity of PS1 mutations could be related to an intracellular mislocalization of the  $\gamma$ -secretase complex composed of these mutants (67, 68). Indeed, in contrary to wild-type PS1 having a broad cellular distribution, mutated PS1 was found to be restricted to endosomal/lysosomal compartments (67, 68). Hence, it was postulated that mutated PS would favor intraneuronal A $\beta$



**Figure 1. APP metabolism.** *A*, represents the  $\alpha/\gamma$ -secretase pathway in which APP is first cleaved by the  $\alpha$ -secretase producing the soluble fragment sAPP $\alpha$  and the membrane-embedded C-terminal fragment, C83 that is then cleaved by  $\gamma$ -secretase to release the cytosolic AICD fragment and p3 peptide, respectively. *B*, represents the  $\beta$  or  $\beta'/\gamma$ -secretase pathway in which APP is first cleaved by the  $\beta$ -secretase at  $\beta$  or  $\beta'$  site to liberate the soluble fragments sAPP $\beta$  or sAPP $\beta'$  and the membrane-embedded C99 or C89 fragments, respectively. Then  $\gamma$ -secretase cleavage releases the cytosolic AICD fragment and A $\beta$  peptides (A $\beta$ 1-40 or A $\beta$ 1-42) or A $\beta$ 11-40, respectively. Note that in physiological conditions,  $\beta$ -secretase mainly cleaves at the  $\beta'$  site, but some FAD mutations shift the cleavage to the  $\beta$ -site to produce more C99. *C*, represents the  $\eta/\gamma$ -secretase pathway in which  $\eta$ -secretase cleavage releases the soluble fragment sAPP $\eta$  and the membrane-embedded  $\eta$ CTF, which is processed by  $\alpha$ - or  $\beta$ -secretase, thus generating A $\eta\alpha$  or A $\eta\beta$  peptides, respectively.

production, which could be particularly toxic (69–71). Moreover, the acidity of endosomal/lysosomal compartments could be less propitious for carboxypeptidase activity, thus leading to the generation of longer and more aggregating A $\beta$  peptides (67) and the acidic pH and high concentrations of A $\beta$  in these compartments could be particularly propitious for A $\beta$  aggregation and thus toxicity.

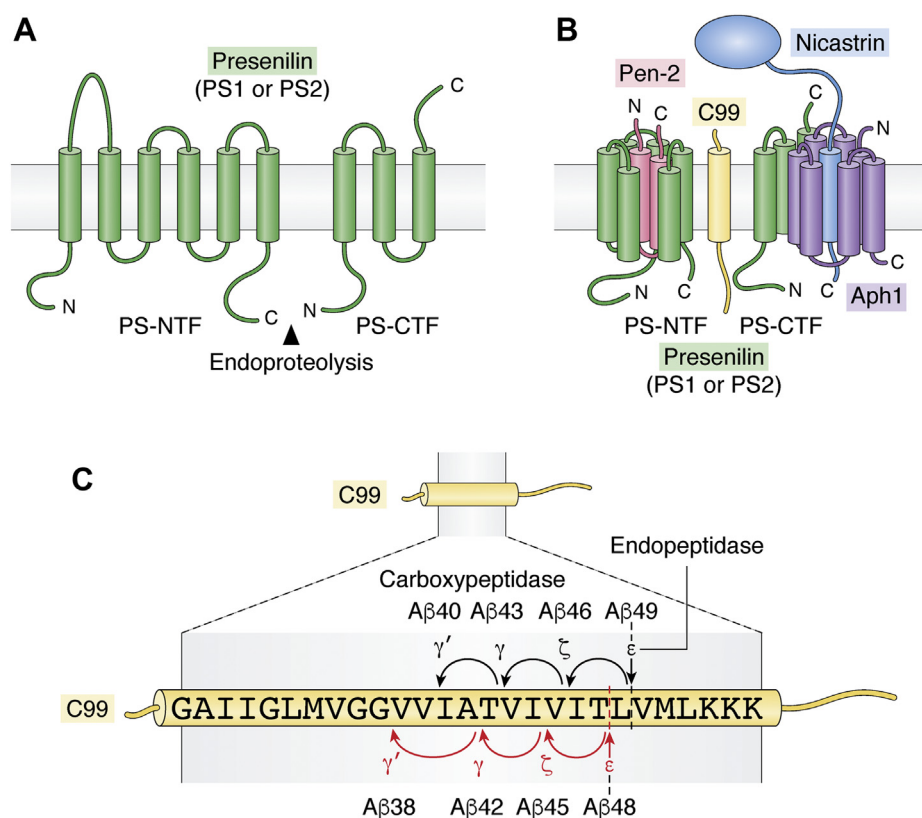
A more recent hypothesis suggests that the pathogenicity of PS mutations could also be linked, or at least partly, to their effect on C99 accumulation. Indeed, although few studies have investigated the effects of FAD mutations on C99 levels, recent studies seem to reveal a consistent increase in C99 in the presence of most pathogenic PS mutants (72–74). This was even the case in neurons derived from pluripotent stem cells (iPSCs) harboring a wide panel of pathogenic PS mutations in the absence of overexpression (73). Whether this effect on C99 is explained by a loss of function (reduced  $\gamma$ -secretase activity) and/or other PS-associated side effects remains unclear. Indeed, accumulating evidence indicates that both PS1 and PS2 are involved in essential cellular processes, independently of their role in  $\gamma$ -secretase, and proposed to be due notably to their function as chaperons and/or control of calcium release from the endoplasmic reticulum (for reviews see (75, 76)). Some of these functions have been suggested to be modified by FAD mutations, although again, many contrasting findings have been reported. Indeed, most studies on noncatalytic functions of PS have been carried out on knockout models, thus in the absence of PS, whereas fewer works have investigated the impact of FAD mutations on these functions. One of the most consistently described noncatalytic functions of PS is

their involvement in the regulation of both autophagy and lysosomal function (75, 76) known also to be involved in C99 degradation (see below). Whether these  $\gamma$ -secretase-independent functions contribute to, or are responsible for, C99 accumulation, in cells expressing PS mutations, remains to be established.

### Physiological function of $\gamma$ -secretase

FAD cases correspond to only a very small subset of patients (about 1%) (77). Thus, both A $\beta$  and C99 accumulation in FAD could reflect an exacerbated process or a dysfunction occurring only in few cases. The situation seems to be different in sporadic AD (SAD) that corresponds to most cases. In SAD, A $\beta$  accumulation is mostly believed to be linked to age-related impairment in A $\beta$  clearance, rather than to an increased A $\beta$  biogenesis. Thus, A $\beta$  should be considered firstly as a physiological product derived from normal processing of APP (78, 79). Although little is still known about the exact physiological role of A $\beta$ , the peptide is known to regulate synaptic activity, to have antimicrobial and tumor suppressing properties (for review see (80)), and to be neuroprotective in some pathological conditions (81–83). Thus, to some extent the failure of clinical trials aimed at either inhibiting A $\beta$  production or neutralizing A $\beta$  once formed could be explained, at least in part, by the abolition of such A $\beta$ -mediated physiological functions.

If considering A $\beta$  as a physiological product, this implies that a main role of  $\gamma$ -secretase should be to generate bioactive molecules involved in important vital processes. Indeed, this point of view fits with genetic evidences indicating that the



**Figure 2.  $\gamma$ -secretase.** *A*, displays a schematic representation of presenilins (PS1 or PS2) composed of nine transmembrane domains and harboring the catalytic core of the complex with two aspartyl residues in the transmembrane domains TM-6 and TM-7. During maturation, presenilin undergoes endoproteolysis, and the resulting N-terminal fragment (PS-NTF) and C-terminal fragment (PS-CTF) remain associated. *B*, represents the multimeric  $\gamma$ -secretase complex composed of four membrane proteins: presenilin (green), nicastrin (blue) composed of a single transmembrane and a large glycosylated N-terminal ectodomain, Aph-1 (purple) composed of seven transmembrane domains, and Pen-2 (pink) composed of two transmembrane domains. The substrate C99 (yellow) interacts with  $\gamma$ -secretase to form an enzyme substrate complex. *C*, represents the successive proteolysis of C99 transmembrane domain by  $\gamma$ -secretase. First, an endoproteolytic cleavage occurs close to the membrane-cytosol interface at two possible  $\epsilon$  sites yielding long A $\beta$  peptides A $\beta$ 48 or A $\beta$ 49, respectively. Then a carboxypeptidase activity of  $\gamma$ -secretase trims A $\beta$ 48 and A $\beta$ 49 each 3 or 4 amino acid at successive  $\zeta$ ,  $\gamma$ , and  $\gamma'$ -sites leading to two A $\beta$  product lines: A $\beta$ 49→A $\beta$ 46→A $\beta$ 43→A $\beta$ 40 and A $\beta$ 48→A $\beta$ 45→A $\beta$ 42→A $\beta$ 38, respectively.

inactivation of  $\gamma$ -secretase in mice is lethal (84) and that the conditional knockout of PS1 leads to neurodegeneration at the adulthood (85). Of course, these observations are probably explained by the numerous, more than 150, other  $\gamma$ -secretase proteolytic targets involved in physiological processes (86). Indeed, similar to APP, most of  $\gamma$ -secretase substrates are type I transmembrane proteins, which undergo “Regulated Intramembrane proteolysis (RIP), a proteolytic process in which  $\gamma$ -secretase-mediated cleavage occurs inside the membrane ending up with the production of an intracellular fragment that shuttles to the nucleus and acts as a transcription factor (87, 88). The seminal example of RIP was described for Notch that undergoes  $\gamma$ -secretase-mediated cleavage, thereby generating the Notch Intracellular Domain (NICD) (89), a key player of embryonic development (90). For instance, this lack of Notch cleavage (91) is believed to explain the lethal embryologic defects observed in PS-invalidated mouse embryos (84). In addition, numerous other substrates (the list is far from being exhaustive) have been reported to undergo RIP including cadherins (N and E Cadh-ICDs) (92), alcadeins (93), CD44 (94), or more recently TREM2 (95). Generally, the generation of the intracellular domain of these proteins is essential in the transcriptional regulation of physiological

processes such as neurite outgrowth, cell adhesion and migration, or synaptogenesis (96, 97). This phenomenon is also described for APP for which the intracellular domain AICD has been found to form a transcriptional active complex with Fe65 and Tip60 (98, 99) that regulates key proteins involved in the control of cell death (p53) (100), A $\beta$  degradation (Neprilysin) (101, 102), as well as tau phosphorylation (GSK3 $\beta$ ) (103, 104). Thus,  $\gamma$ -secretase cleavage should be considered as a necessary step in the formation of both A $\beta$  and AICD. Besides this role,  $\gamma$ -secretase cleavage has also been proposed to behave as a “membranous proteasome” essential for the inactivation of the buildup of toxic membranous stubs (105). It remains unclear, whether this “proteasome-like activity” is nonselective and inactivates all substrates equally, or if some substrates are cleaved by specific protease complexes. Recent evidences seem to indicate at least some substrate specificity and that not only the exact protein composition of the secretase complex, but also the cellular context, subcellular localization, and the presence of nonessential cofactors (106) can be key determinants for this control. For instance, protease heterogeneity has been shown to be related to the exact protein composition of the protease and alternative splicing of Aph1 leads to further complexity (107, 108). For instance, in

**Table 2**  
C99-associated toxicity in AD mice models and human brain

Mouse models	Mutations	C99 accumulation-associated pathology	References
3xTgAD 2xTgAD	APP <sup>K670N,M671L</sup> , PS1 <sup>M146V</sup> , MAPT <sup>P301L</sup> APP <sup>K670N,M671L</sup> , MAPT <sup>P301L</sup>	Endolysosomal and autophagic dysfunction Inflammation, LTP alterations Mitochondrial pathology	(128, 137, 139, 163, 168)
5xFAD APP/PS1	APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> , APP <sup>V717I</sup> , PS1 <sup>M146L,L286V</sup> APP <sup>751<sub>K670N,M671L</sub></sup> , PS1 <sup>G384A</sup>	Mitochondrial pathology Accumulation in MAMs	(224) (155)
APPE663Q TgCRND8 J20 Knock-in APP <sup>NL</sup> APP <sup>NL-F</sup> and APP <sup>NL-G-F</sup> APP <sup>Hm/Hm</sup>	APP <sup>E663Q</sup> APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> APP <sup>KM670/671NL</sup> , APP <sup>V717F</sup> APP <sup>KM670/671NL</sup> , APP <sup>KM670/671NL,L1716F</sup> APP <sup>KM670/671NL,E1716F,E693G</sup> hA $\beta$ sequence (G67/68,F681,Y,R684H) Various APP and PS mutants	Lysosomal dysfunction, inflammation Brain network alterations Brain network alterations Nd Nd	(131) (129, 227) (130) (132–134)
Human iPSCs		Lysosomal dysfunction	(143)
Down syndrome fibroblasts	Ts65DN mice or human patients	Endosomal pathology Endosomal pathology lysosomal dysfunction Axonal transport defects	(140, 228–230)
Human brain	FAD SAD	Nd Correlation with neuronal vulnerability	(126, 137, 138, 175)

APP, amyloid precursor protein; FAD, familial Alzheimer disease; IPSC, induced pluripotent stem cells; LTP, long-term potentiation; MAPT, microtubule-associated protein tau; nd, not determined; PS, presenilin; SAD, sporadic Alzheimer's disease.

mice the knockout of the 3 Aph1 subunits led to an important increase in some, but not all, membrane-bound fragments and APP-dependent neurodegeneration (107, 108). Hu and colleagues demonstrated that nicastrin is required for APP processing but not Notch processing, whereas Aph1 is necessary for the processing of both APP and Notch (109). Selectivity also seems to be determined by the subcellular localization of the protease, since, as described above, PS1- or PS2-containing complexes do not have the same cellular localization and do not cleave APP identically (67, 68). Interestingly, mutations in PS1 change the localization toward that of PS2-containing complexes (67).

### Challenges and complications of targeting $\gamma$ -secretase in AD

After the discovery of  $\gamma$ -secretase, a huge amount of research was carried out to develop potent and bioavailable  $\gamma$ -secretase inhibitors (GSIs), but the clinical trials based on these inhibitors all failed (Table 1). Indeed, none of them led to improvement of AD-linked cognitive decline and even sometimes they worsened them. In most of these trials, A $\beta$  levels in plasma or cerebrospinal fluid (CSF) were significantly decreased, thus clearly indicating that a reduction in total A $\beta$  was not sufficient to restore cognitive function. This was the case for Semagacestat, the first GSI tested in late-stage clinical trials for AD, which was reported to reduce A $\beta$  levels by more than 60% in the plasma (110), and new A $\beta$  synthesis was decreased by more than 80% in CSF (111). More alarming, many trials were readily stopped because of increased risk of developing skin cancer and infections, which was supposed to be tightly linked to a defective Notch signaling in the presence of the inhibitors (112). Therefore, several groups started to screen for drugs having a higher affinity for APP than toward Notch, such as Avagacestat, initially reported to be 140-fold more selective for APP than Notch (113), although its Notch sparing ability remains controversial (114, 115). Avagacestat led to a 40% A $\beta$  reduction in CSF (Table 1), but demonstrated similar side effects in clinical trials (116). Therefore, due to the huge number of different  $\gamma$ -secretase substrates, it has appeared difficult to target specifically and exclusively the  $\gamma$ -secretase-mediated APP cleavage.

Furthermore, data from preclinical trials proposed that the lack of efficacy, or even worsening, of cognitive function in these trials was also linked to the progressive accumulation of APP-CTFs in the presence of GSIs (117). To avoid such effects, therapeutic development became focused on  $\gamma$ -secretase modulators (GSMs) that were expected to be safer, since they interact with  $\gamma$ -secretase complex through the allosteric binding site, thereby modifying the enzyme activity but not blocking it (118). The concept of  $\gamma$ -secretase modulation was discovered with some nonsteroidal anti-inflammatory drugs (NSAIDs) such as flurbiprofen, indomethacin, and ibuprofen, which are considered as GSMs, because they induce conformational changes in PS1 and shift the cleavage of C99 toward shorter A $\beta$  species such as A $\beta$ 37 and A $\beta$ 38 (16, 119). In that way, GSMs do not lead to increased APP-CTFs levels. Indeed,

in AD animal models, the chronic treatment with GSMs did not lead to the worsening of cognitive function observed with GSIs (117). Moreover, GSMs are also safer because they reduce the level of A $\beta$ 42 and increase shorter A $\beta$  peptides without affecting Notch signaling (120, 121). Nonetheless, the first GSMs tested in clinical trials, Rofecoxib (122), Tarenflurbil (123) or Naproxen (124), did not show efficacy, but the lack of effects seemed to be related to a very poor blood–brain barrier crossing ability of these drugs (Table 1).

Thus, taken together,  $\gamma$ -secretase-based strategies have so far been unsuccessful, even if GSMs seem to be more promising (125). Still, if considering  $\gamma$ -secretase as a C99-inactivating enzyme, we believe that one should carefully question the validity of targeting this enzyme.

### **C99 is toxic and $\gamma$ -secretase inhibitors potentiate pathogenic phenotypes**

Emerging data propose a pathological role of the intraneuronal accumulation of C99 (Table 2). Indeed, a very recent work showed that the accumulation of C99, rather than that of A $\beta$ , correlates with neuronal vulnerability in AD-affected patients (126). To distinguish between C99 and A $\beta$  *in situ*, which can be very challenging because the two of them have common epitopes, the group of Drs Bustos and Greengard developed a sophisticated technique adapted from the proximity ligation assay (PLA) and using both N- and C-terminal directed antibodies. This link between neuronal vulnerability and C99 in human AD was an important observation previously supported by many animal models. The first evidence of C99 accumulation in AD mouse models came from studies on the widely used 3xTg-AD model (APP<sub>swe</sub>, Tau<sub>P310L</sub>, PS1<sub>M146V</sub>) developed by the group of La Ferla (127). In the original work, the authors used N-terminal A $\beta$  antibodies and claimed that these mice harbor intracellular A $\beta$  accumulation (127). However, years later our own work, using different and complementary approaches, indicated that this accumulated material corresponded to C99 and not to A $\beta$  (128). Indeed, as stated above, it is challenging to discriminate between A $\beta$  and C99 *in situ*. Hence, only a detailed characterization using N- and C-terminal antibodies, pharmacological treatments ( $\beta$ - and  $\gamma$ -secretase inhibitors), and/or genetic approaches (as PS mutations) can allow this discrimination. Most and even recent studies do not provide this detailed characterization, which may explain the lack of information concerning C99 in the literature. In the 3xTgAD model, C99 accumulates early and much before A $\beta$  can be detected (128). Of importance, C99 accumulation was found to also occur in many other transgenic models including Tg-CRND8 (129) and JA20 (130) mice carrying the APP<sub>swe</sub> mutation, known to increase C99 production, but also in mice displaying the APP<sup>E693Q</sup> Dutch mutation (131), in which a C99 overproduction is not expected to occur. Interestingly, and importantly, C99 accumulation also occurs in the knock-in models APP-NL and APP-NL-F, harboring the Swedish (KM670/671NL) mutation alone (APP-NL) or in combination with the I706F mutation (APP-NL-F), respectively (132, 133) (Table 2) indicating that C99

accumulation was not just an artificial phenotype linked to APP overexpression, as earlier described for other events taking place in transgenic mouse models that sometimes express very high levels of APP (132). The presence of the Swedish mutation could indeed, at least to some extent, explain the C99 accumulation in these knock-in mice. However, recent studies clearly showed that humanizing APP (substituting the three amino acids G676R, F681Y, and R684H) in mice or rats is sufficient to increase C99 levels (134–136). In animals displaying rodent APP, the protein was found to be mainly processed by  $\alpha$ -secretase, thus generating almost only C83, whereas the presence of human APP increases  $\beta$ -secretase cleavage and leads to enhanced C99 levels (134). These data thus reveal a different APP processing of human and rodent APP, which could explain the higher levels of C99 in human as compared with rodents (137) and may be one of the reasons for the C99 accumulation observed even in SAD cases (126, 137, 138). Overall, these findings ruled out the possibility that it could be “model specific” but rather suggest that it should be a common and early alteration in AD.

In AD mouse models, such as in 3xTg-AD mice, C99 accumulation was described to be both the cause and the consequence of endosomal perturbations, ultimately leading to failure of lysosomal and autophagic processes (139). A similar link between C99 accumulation and lysosomal dysfunction was described in the mouse bearing the Dutch mutation APP<sup>E693Q</sup> (131). Moreover, in fibroblasts from Down syndrome patients or from the mouse model Ts65Dn, C99 were found to induce enlargement of Rab5 positive early endosomes, aberrant endocytosis, and impaired endosomal transport (140). Interestingly, these alterations were fully A $\beta$ -independent, since they were rescued by  $\beta$ -secretase inhibitors or partial BACE1 genetic depletion and were enhanced by GSIs (139–142). Of utmost interest, C99 accumulation has also been associated to endolysosomal dysfunction in human cellular AD models. Indeed, the earlier mentioned transcriptomic analysis of induced pluripotent stem cells (iPSCs) harboring AD-related APP or PS1 mutations indicated similar changes in endocytosis-associated genes (73). Strikingly, these mutations brought divergent data concerning A $\beta$ , but they all displayed increases in C99 levels as well as endosomal dysfunction (73). The use of notably a BACE inhibitor, GSMs and GSIs, showed that endosomal pathology was linked to C99 and not to the changes in A $\beta$  (73). Resembling data were obtained in another study using iPSCs-derived neurons obtained from AD patients harboring APP or PS1 mutations, which displayed both C99 accumulation and dysfunction of the endosomal–lysosomal network (143). As a difference to the work of Kwarts and colleagues, mutations in APP but not in PS1 led to early endosomal (Rab5 associated) pathology, but all mutations led to enlarged late endosomes and defective lysosomal degradation. These effects were APP-dependent, reversed by BACE inhibition, and exacerbated by GSIs. These results agree with the consistent data linking C99 to a dysfunction in the endolysosomal network occurring even in the presence of physiological APP levels (144). Whether C99 accumulation in these systems is linked to the partial loss of



function of  $\gamma$ -secretase or to other PS but  $\gamma$ -secretase-independent mechanisms, such as impaired lysosomal-autophagic degradation, as discussed earlier in this review, remains to be established. Related to endolysosomal dysfunction, the pathogenicity of C99 could also be due to its propensity to aggregate and to spread through exosomes, small vesicles secreted from cells (145). Indeed, exosomes originate from endosomes and C99 is detected in these vesicles (145–147). Our recent work showed the possible presence of C99 existing as oligomers (C99 homomers and heteromers composed of C99/C83) in exosomes, the levels of which were drastically enhanced upon  $\gamma$ -secretase inhibition (145). The latter statement is of importance due to the proposed role of exosomes in prion-like transmission of neurotoxic molecules (148).

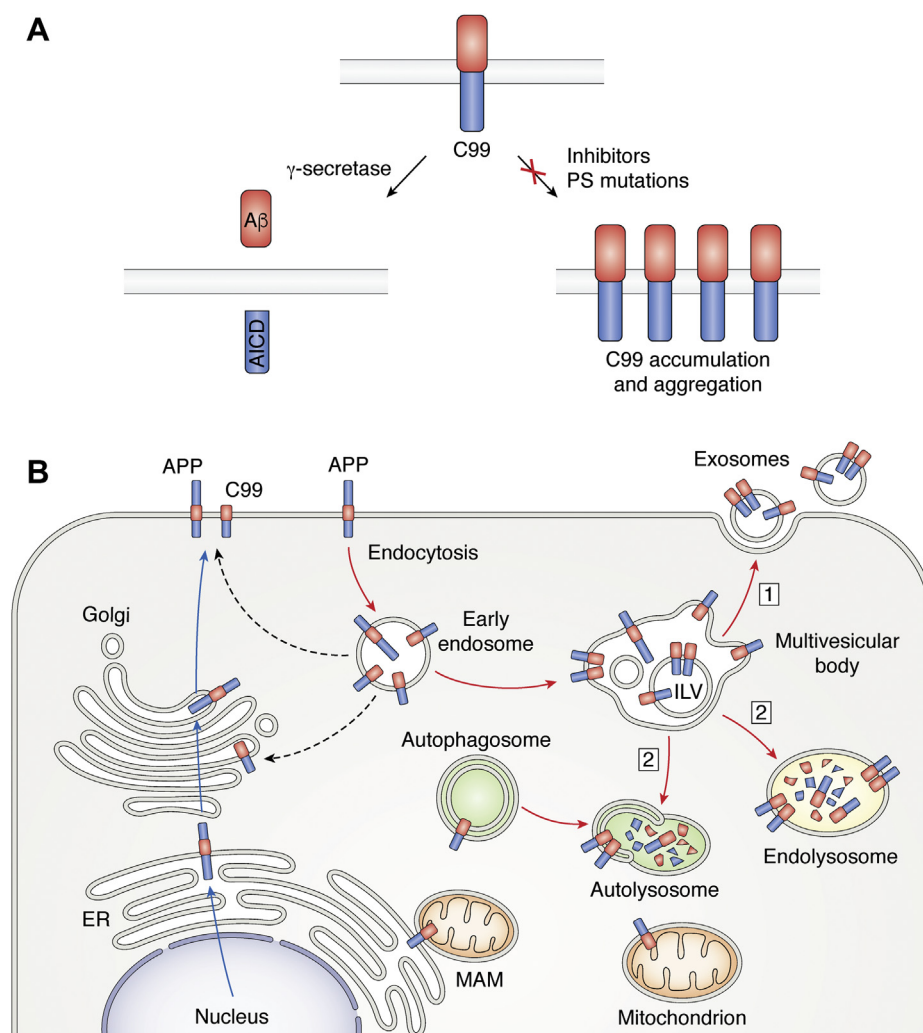
In addition to endosomal and lysosomal alterations, mitochondrial dysfunction is a key feature of AD with altered mitochondrial potential, increased levels of reactive oxygen species (ROS) (149–151), as well as a defect in clearance of abnormal mitochondria (mitophagy) (152, 153). Our recent work proposed that in cellular models, C99 can trigger all these alterations (137). In agreement, mitochondrial structure alterations and mitophagy defects were observed in young preplaque 3xTg-AD mice, as well as in virus-induced C99 expressing mice (AAV10-C99 mice) and notably following GSI treatment (137). Other studies have demonstrated C99 accumulation in mitochondria-associated membranes (MAMs) (154, 155) and have proposed its contribution to neutral lipid accumulation (155) and cholesterol trafficking (156).

Neuroinflammation is also a key feature of AD, but little is known about its molecular triggers. The contribution of C99 to neuroinflammation was proposed as early as in 1998 in mice, in which the intracerebroventricular injection of recombinant C99 (CT105) was found to induce reactive gliosis and neurodegeneration (157). CT105 was also found to interact with inflammatory cytokines and produce a synergistic effect on working memory (158). Two more recent works also proposed a link between intraneuronal accumulation of C99 and astrocytic activation and astrogliosis in 3xTgAD and APP<sup>E693Q</sup> mice, respectively (128, 131). Furthermore, in preplaque TgCRND8 mice, C99 was shown to correlate with a TNF $\alpha$  augmentation and microglial activation (129).

Does C99 accumulation also trigger functional and cognitive alterations? It is well established that AD is characterized by a hippocampal altered long-term potentiation (LTP) and depression (LTD) (159), as well as network activity dysfunctions (160). All of these electrophysiological signatures are linked to memory consolidation and learning ability and appear altered before senile plaques and neurodegeneration in human AD (161) and many mouse models. The first evidence of a neurotoxic effect of C99 on synaptic function was demonstrated years ago in a transgenic mouse model expressing directly the C99 fragment (162). These mice were found to develop both LTP and memory alterations, as well as neuroinflammation and neuronal loss. Our own recent studies in AAV10-C99 mice showed that LTP was significantly reduced in preplaque mice and that GSIs did not reverse LTP alterations (139). Synaptic alterations also seemed to be

temporarily and spatially correlated to C99 accumulation in 3xTgAD mice (163) and in the Danish dementia mouse model, in which a deficiency in the BRI2 protein leads to increased APP levels and in which LTP alterations and memory defects were rescued by the pharmacological inhibition of  $\beta$ -secretase, but not  $\gamma$ -secretase (164). Hippocampal network oscillations, as well as  $\theta$ / $\gamma$  coupling alterations, were reported at early “A $\beta$ -free” stages in the Tg-CRND8 mouse (165). In this study, these early network defects occurred in C99-accumulating regions and were fully rescued by  $\beta$ -secretase inhibition (130). In contrast, another recent study reported an absence of rescue of network alterations by  $\beta$ -secretase inhibition in the J20 model, although the levels of both C99 and A $\beta$  were clearly reduced (133). These data show that synaptic function is governed by complex mechanisms that may be difficult to rescue. First of all, in these studies  $\beta$ -secretase inhibition led to a decrease in C99 and A $\beta$ , but it also led to a concomitant increase in C83 that maybe could contribute to these alterations, although this was not discussed by the authors. Second of all, and maybe more importantly, the lack of rescue by BACE1 inhibitors could be due to the suppression of important cell signaling pathways involved in synaptic function, due to the inactivation of other BACE1 substrates, such as Neuregulin-1 and Sez6 (166, 167). In the same way, studies investigating the role of C99 accumulation in AD-related memory impairment in mouse AD models have reported conflicting data. In 3xTgAD mice, C99 accumulation seems to trigger both electrophysiological hippocampal defects and mild cognitive dysfunction (163, 168). Similar defects were observed in CT105-expressing mice (162). However, the studies from knock-in models seem to argue for an A $\beta$ -rather than a C99-dependent contribution to synaptic alterations. In fact, the two knock-in models APP-NL and APP-NL-F, harboring the Swedish (KM670/671NL) mutation alone (APP-NL), or in combination with the I706F mutation (APP-NL-F), respectively, both accumulate C99 (132), but only the APP-NL-F, displaying higher A $\beta$  levels, develops late-stage cognitive dysfunction (132, 169). Moreover, the knock-in mouse APP-NL-G-F harboring a third mutation, the E693G Arctic mutation, which generates a particular high level of oligomerized A $\beta$ , displays cognitive dysfunction at a much younger age. Thus, taken together, these observations could suggest either that only A $\beta$  is toxic or that A $\beta$  and C99 contribute differently to the pathology. Whereas A $\beta$ , as a soluble peptide, could be expected to act mainly outside cells, the toxic effect of membrane-embedded C99 should be intracellular. It is possible that intraneuronal C99 is a trigger of a more slowly developing pathological process that might be more prominent in transgenic mice that display higher levels of C99 than knock-in mice.

In 3xTgAD mice, these electrophysiological defects are not only accompanied by alterations in spatial learning and memory, but are also associated to a decrease in spontaneous activity that is reminiscent to apathy (168), a phenotype considered as an early neuropsychiatric symptom in AD patients (170, 171). Several observations suggested that this apathy-like behavior could be linked to C99 accumulation.



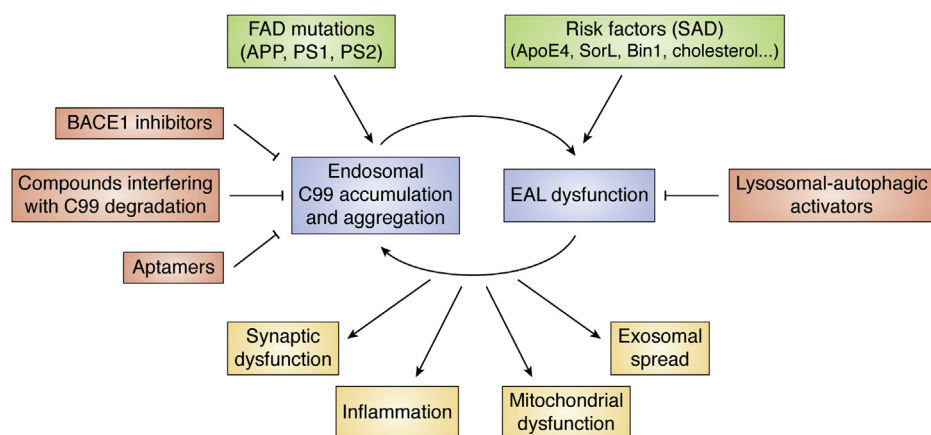
**Figure 3. Schematic models for C99 accumulation.** A, displays a schematic model of C99 fate in the absence or presence of  $\gamma$ -secretase cleavage. In physiological conditions,  $\gamma$ -secretase cleavage of C99 (blue/red sticks) leads to the generation of A $\beta$  and AICD, whereas when  $\gamma$ -secretase activity is impaired (inhibitors or PS mutations), C99 accumulates and aggregates. B, displays a schematic view of C99 generation and accumulation in conditions of blocked  $\gamma$ -secretase. APP (long blue/red bar) matures and traffics through the Golgi to the plasma membrane (blue arrows), where a small part of it is processed by  $\alpha$ -secretase (non-amyloidogenic pathway). APP that escapes  $\alpha$ -secretase-mediated cleavage is endocytosed into early endosomes and some of it is recycled to the plasma membrane, either directly or through the Golgi network (open black arrows). Within early endosomes, some APP is cleaved by  $\beta$ -secretase generating C99 (short blue/red bar), which then can undergo  $\gamma$ -secretase cleavage, thereby releasing the A $\beta$  peptide and AICD. Some C99 escapes from  $\gamma$ -secretase cleavage and is incorporated in ILVs (intra luminal vesicles), which are either released as exosomes after the fusion of multivesicular bodies (MVBs) with the plasma membrane (1) or degraded after their fusion of MVBs with lysosomes or with autophagosomes, which then fuse with lysosomes (2). The presence of APP and PS mutations or  $\gamma$ -secretase inhibitors and/or lowered lysosomal degradation lead to lysosomal, autolysosomal, and exosomal C99 accumulation. C99 has also been found to accumulate in MAMs and mitochondria.

This phenotype not only appears at a stage much before A $\beta$  could be detected, but it similarly occurs in a 2xTg-AD model (harboring APP<sup>Swe</sup> and Tau P30L), which was found to accumulate identical amounts C99 to the 3xTgAD mouse, but which display very low A $\beta$  levels even at late stages (168).

### Is $\beta$ -secretase a good drug target?

BACE1 cleavage of APP represents the rate-limiting step of A $\beta$  production, and targeting this enzyme would provide advantage to prevent production of both A $\beta$  and C99. To date, there is no evidence of AD-linked mutations on BACE1, but a clue of an indirect importance of BACE1 in AD came from genetic studies showing that APP mutations lying close to the  $\beta$ -secretase cleaving site strongly affect the cleavage by this

enzyme and can either cause early-onset AD (the Swedish and Leuven mutations) or be protective (the Icelandic mutation). BACE1 has also been shown to have a role in SAD as its expression was reported to be increased in AD patients. Thus, BACE1 protein was found to be increased 3-fold in cortical areas of AD patients as compared with age-matched controls (172), and these data were confirmed at the mRNA level in the frontal cortex of SAD patients (172, 173). Yang and colleagues also showed a significant elevation of BACE1 in temporal cortex and hippocampal samples from a cohort of clinically diagnosed and neuropathologically confirmed AD patients (174). This study used fluorimetric analysis assays and *in vitro* proteolysis to confirm that this increase in BACE1 expression led to enhanced functional enzyme. At that stage, most of the



**Figure 4. C99 toxicity.** C99 accumulation can be a consequence of either the presence of FAD mutations or a lowered degradation (possibly linked to risk factors). C99 firstly accumulates in endosomes and can itself be a direct cause of a dysfunction of the endosomal–lysosomal–autophagic (EAL) degradation pathway, thus leading to a vicious pathological cycle, in which more C99 accumulates, aggregates, and becomes neurotoxic by causing synaptic dysfunction, inflammation, mitochondrial dysfunction, and exosomal spread. Therapeutic strategies aimed at reducing C99 levels could be through either BACE1 inhibitors and/or BACE1 aptamers, which should be expected to reduce its production or autophagic/lysosomal activators or other drugs interfering with C99 degradation, which should increase its degradation.

studies attempted to correlate BACE1 expression to A $\beta$  load. However, some later studies analyzed C99 levels and reported also an increase in both FAD and SAD brains (137, 138, 175). Of most interest, the recent work from Pulina and colleagues quantifying both C99 and A $\beta$  in either controls, mildly or severely-affected AD brains, showed that C99 accumulates specifically in vulnerable neurons and correlated with the degree of cognitive alterations, whereas A $\beta$  load was increased in both vulnerable and resistant brain regions in AD (126). The latter data are to be put back into context with the consistent former observations that A $\beta$  expression and distribution within brain areas do not fully match neuronal loss, cognitive defects severity, and dementia AD progression (176–178). It should be emphasized that two recent studies documented that BACE may inactivate an intravesicular domain of APP, thereby exacerbating glutamate release (179), a process enhanced in a rat knock-in model bearing the Swedish mutation (135).

As stated above, BACE1 mainly cleaves APP at the  $\beta'$  site to generate C89 and therefore can be considered to be protective. In addition, BACE1 is also able to cleave A $\beta$ 40 and A $\beta$ 42 into the nonamyloidogenic (180) and neuroprotective (181) A $\beta$ 34. A recent study also proposed another neuroprotective role of BACE1, since the inhibition of this enzyme was found to enhance the levels of both  $\eta$ -secretase and A $\eta\alpha$ , the latter was proposed to be neurotoxic (27). Thus, in AD, the shift from a beneficial to a deleterious BACE1 cleavage could be due to the age-dependent augmentation of BACE1 expression occurring in sporadic AD or to the expression of a subset of pathogenic mutations in FAD, thereby enhancing C99 levels above physiological threshold (32).

All clinical trials targeting BACE-1 that have entered in phase III have been reported to decrease significantly and dose-dependently A $\beta$  levels in the brain, plasma, or CSF (Table 1). However, all of them have been terminated mostly due to reported toxicity. Indeed, many studies have

demonstrated serious adverse effects such as an abnormal elevation in liver enzymes and clinical worsening (Table 1). The reasons for these side effects observed in human trials are not well established, but could be related to the high number of BACE1 substrates being involved in vital functions. BACE1 is known to cleave nearly 40 different neuronal membrane proteins (182) and some of these substrates, such as Sez6 or neuron myelination like neuregulin-1, are known to be essential for synaptic function (183). Indeed, several studies reported that BACE1 inhibition triggers synaptic and cognitive dysfunctions (166, 167). BACE1 inhibitors could also cross over with not only its homologue BACE2, but also with cathepsin D, another aspartyl protease (184, 185). Inhibition of cathepsin D would be expected to be highly deleterious because of its role in lysosomal degradation. Of note, cathepsin D is one of the key enzymes involved in C99 degradation (139, 186) and would therefore also be expected to slow down the degradation of C99 (139, 186). It is therefore of high importance to identify inhibitors that target BACE1 but no other acidic proteases. Moreover, ideally these inhibitors should specifically decrease the processing of APP but not that of other BACE1 substrates.

Until yet, one of the main obstacles of BACE1 inhibitors is linked to their limited drug availability due to their peptidic feature and high molecular size. Small molecules may be less efficient than APP to fill the large substrate pocket, but higher sized molecules may display reduced bioavailability. These drugs need to cross the brain barrier, but also the cell membrane to reach endosomes, which are the main sites for  $\beta$ -secretase (36). Great advances in the field have been the recent development of nonpeptidic inhibitors with promising pharmacological properties, but still these drugs have not demonstrated convincing effects on cognitive function. This has been the case for the clinical trial with the small-molecule  $\beta$ -secretase inhibitor Umibecestat, which was administered to preasymptomatic carriers of APOE4 and led to more than 90% reduced A $\beta$  levels

in CSF but was stopped due to deleterious cognitive and side effects (187).

### Alternative strategies to decrease C99 buildup

Besides being processed by  $\gamma$ -secretase, a part of C99 is eliminated by degradation through the autophagic/lysosomal pathway (92, 143) and in some AD models, C99 accumulation is a consequence of a defective lysosomal clearance (139). Endosomal–lysosomal–autophagic dysfunction is indeed one of the earliest and major features of AD and has been proposed to be caused by both FAD mutations (as described above) and risk factors associated with SAD (for review see (188)). Thus, the activation of this degradation pathway could be expected to reduce C99 levels and to be beneficial. Rapamycin, a specific inhibitor of mTOR and a well-known inducer of autophagy, has been reported to ameliorate tau pathology, reduce amyloid- $\beta$  (A $\beta$ ) deposition, and improve cognition in preclinical studies (189, 190). However, rapamycin, as well as other autophagic activators, enhances autophagy without increasing lysosomal function that may be associated with an accumulation of nondigested autophagic vacuoles. Thus, a better way to enhance degradation could be to activate both autophagy and lysosomal function by overexpressing or pharmacologically activating the transcription factor EB (TFEB), the main regulator of the lysosomal/autophagic degradation pathway (191). TFEB activates the different steps of autophagy, from its initiation to the fusion of autophagosomes with lysosomes, but it also stimulates the biogenesis of lysosomal hydrolyses and regulates lysosomal acidification (191). Indeed, in animals, TFEB activation has been found to efficiently decrease C99 levels (192–194) and to ameliorate cognitive function, whereas it does not seem to produce any harmful effects in wild-type animals (143). The recent findings concerning TFEB activation by a curcumin analog are promising, because of the lack so far of specific TFEB-activating molecules. Earlier studies have proposed cyclodextrin, a modifier of cholesterol efflux used for the treatment of Niemann–Pick disease, as a TFEB activating drug. However, a recent work has demonstrated conflicting data concerning this molecule (195). Whereas short-term treatment was found to lead to activated autophagic degradation, long-term treatment had an opposite effect probably caused by the effect of cyclodextrin on membrane lipids (195). In addition, pharmacological treatments such as acidic nanoparticles, PLGA (poly lactic-co-glycolic acid, called acidic nanoparticles (196) could constitute an alternative way to increase lysosomal function. These nanoparticles localize to lysosomes, where they degrade, and subsequently release their acidic components to acidify the local lysosomal environment (197). Acidic nanoparticles have notably been proven efficient in cellular and animal models of Parkinson's disease (197).

Finally, recent works have proposed novel mechanisms involved in C99 degradation and have provided new potential drug targets. For instance, it was demonstrated that the selective phosphorylation of presenilin 1 at ser367 could be a way to increase autophagic degradation of C99. Indeed, this

phosphorylation of PS1 was found to facilitate autophagic–lysosomal fusion, an effect that was mediated through the interaction of phosphorylated PS1 with Annexin 2 and snare proteins. These findings therefore proposed that drugs designed to selectively increase phosphorylation of presenilin1 at ser367 could be a way to circumvent C99 accumulation (198, 199). Other works have shown that lysosomal/autophagic degradation of C99 can be promoted by its binding to the adipocyte differentiation protein AMAP, which could therefore also constitute a new drug target (200, 201). The  $\gamma$ -secretase complex, often *via* the presenilins, binds to many endogenous proteins, which will influence  $\gamma$ -secretase cleavage in distinct ways (202, 203).  $\gamma$ -Secretase has also been found to bind APP-CTFs, thereby stabilizing them for nonspecific degradation (204). A recent study proposed that ILE1, a member of the FAM3 superfamily, interferes with these chaperone properties and thereby enhances C99 degradation (204). ILE1 and molecules having similar properties may therefore also be plausible targets for future therapies.

Besides the strategies aimed at reducing C99 levels by enhancing its degradation, two other alternative strategies are possible. Firstly, the design of aptamers (so-called chemical antibodies) and the successful outcomes of their use in pathological conditions (205) allow to envisage this strategy to preclude side effects of BACE1 inhibitors on its physiological substrates. These chemical probes should be designed to prevent direct physical interaction of BACE1 with its targeted APP sequence in order to selectively hamper BACE1-mediated production of C99. Secondly, C99 levels could be reduced by repressing the transcriptional of APP. Indeed, a recent study has showed such effects of the compound Posiphen, which normalized C99 levels, but also reversed early endosome enlargement in Ts65Dn mice (206). This study not only reaffirms the link between C99 and endosomal perturbation but also indicates that the upstream regulation of its precursor APP could be a potential track to circumscribe C99 accumulation and subsequent cellular alterations.

### Conclusion and perspectives

Taken together, two lines of conclusions can be drawn from the above-described observations. First, studies on a vast number of  $\gamma$ -secretase substrates have mostly if not always shown that their catalytic processing leads to the conversion into bioactive fragments, the generation of which governs a large spectrum of vital functions (96, 97). This is probably also the case of  $\gamma$ -secretase cleavage on APP leading to the generation of physiological levels of A $\beta$  and AICD. Second, a network of consistent evidence suggests that, first, both FAD and SAD are associated with C99 accumulation; second, that C99, independently of A $\beta$ , behaves as an early pathogenic trigger in AD, and third, that all C99-associated biochemical, anatomical, and cognitive alterations are drastically potentiated by  $\gamma$ -secretase inhibitors (Fig. 3). C99 accumulation is believed to begin mainly in endosomes and has been shown to be a direct cause of endolysosomal network dysfunction, thus leading to a vicious pathological cycle, in which C99

accumulates, aggregates and becomes neurotoxic by causing synaptic dysfunction, inflammation, mitochondrial pathology, and exosomal spread (Fig. 4). The causes of this AD-related C99 accumulation could be an increased production (presence of APP mutations in FAD or enhanced BACE expression in SAD), a loss of  $\gamma$ -secretase cleavage (FAD PS and APP mutations) or a decreased degradation due to a defective lysosomal-autophagic degradation (early feature linked to FAD mutations and risk factors in SAD).

Our conclusion reconciles genetic grounds indicating a strong link between  $\beta$ APP and AD pathology and the systematic failure of A $\beta$ -centric clinical assays. So far, evidence points to a particularly important role of C99 at early stages of AD, but these findings do not preclude the undoubted role of A $\beta$  and particularly at later stages. The toxic effect of C99 could also be completely different from that of A $\beta$ . While the membrane-embedded C99 could affect mostly intracellular pathways, A $\beta$  could be expected to act mostly extracellularly. We propose that  $\gamma$ -secretase is an important C99-inactivating enzyme and that the consistent failure, and sometimes the deleterious worsening, of clinical trials based on  $\gamma$ -secretase inhibition could be linked, at least partly, to the impairment of C99 inactivation. GSMs are certainly more promising than GSIs, because they are not expected to increase C99 levels. However, the lack of C99 inactivation in the presence of these molecules still might be problematic. Future studies are needed to clarify this issue.

If one considers the fate of C99, secretases could overall be seen either as direct C99 inactivators, such as  $\gamma$ -secretase as stated above,  $\alpha$ -secretase that cleaves C99 to generate C83 (207), and even  $\beta$ -secretase that mainly cleaves  $\beta$ APP at the  $\beta'$  site, thereby precluding C99 production (Fig. 1B). In that sense, our point of view is that therapeutic strategies targeting secretases would not be the best way to tackle AD. Indeed, besides the complexity and the high number of substrates other than APP, inhibiting these cleavages would also impair C99 degradation.

Thus, in conclusion, we here suggest that  $\gamma$ -secretase is a crucial enzyme involved in not only the production of physiological bioactive fragments but also the inactivation of neurotoxic C99. We propose alternative strategies aiming to reduce the levels of C99, either by interfering with its production or by promoting its degradation (Fig. 4).

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**Abbreviations**—The abbreviations used are: A $\beta$ , amyloid  $\beta$ ; AD, Alzheimer’s disease; AICD, APP intracellular domain; APH, anterior pharynx defective; BACE,  $\beta$ -site APP cleaving enzyme;  $\beta$ APP, amyloid precursor protein; CSF, cerebrospinal fluid; CTF, C-terminal fragment; EAL, endosomal-lysosomal-autophagy; ER, endoplasmic reticulum; FAD, familial Alzheimer’s disease; GSM,  $\gamma$ -secretase modulators; LTP and LTD, long-term potentiation and depression; MAM, mitochondrial membrane associated; NCT, nicastrin; PEN, PS enhancer; PS, presenilin; ROS, reactive oxygen species; SAD, sporadic Alzheimer’s disease; TFEb, transcriptional factor EB.

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