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# Does intraneuronal accumulation of carboxyl terminal fragments of the amyloid precursor protein trigger early neurotoxicity in Alzheimer's disease?

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## Abstract:

**Background:** Alzheimer's disease (AD) is associated with extracellular accumulation and aggregation of amyloid  $\beta$  ( $A\beta$ ) peptides ultimately seeding in senile plaques. Recent data show that their direct precursor C99 ( $\beta$ CTF) also accumulates in AD-affected brain as well as in AD-like mouse models. C99 is consistently detected much earlier than  $A\beta$ , suggesting that this metabolite could be an early contributor to AD pathology. C99 accumulates principally within endolysosomal and autophagic structures and its accumulation was described as both a consequence and one of the causes of endolysosomal-autophagic pathology, the occurrence of which has been documented as an early defect in AD. C99 was also accompanied with C99-derived C83 ( $\alpha$ CTF) accumulation occurring within the same intracellular organelles. Moreover, both  $\alpha$ - and  $\beta$ -CTFs can dimerize, thereby leading to the generation of higher molecular weight CTFs, which were immunohistochemically characterized *in situ* by means of aggregate-specific antibodies. Here, we discuss studies demonstrating a direct link between the accumulation of C99 and C99-derived APP-CTFs and early neurotoxicity. We discuss the role of C99 in endosomal-lysosomal-autophagic dysfunction, neuroinflammation, early brain network alterations and synaptic dysfunction as well as in memory-related behavioral alterations, in triple transgenic mice as well as in newly developed AD animal models.

**Conclusion:** this review summarizes current evidence suggesting a potential role of the  $\beta$ -secretase-derived APP C-terminal fragment C99 in Alzheimer's disease etiology.

**Running title:** APP CTFs in Alzheimer's disease

**Keywords:** Alzheimer's disease, C99, C83, C-terminal APP fragments, dimerization, endolysosomal-autophagic dysfunction, synaptic dysfunction, brain network alterations, memory-related behavior, animal models.

## Introduction

Alzheimer's disease (AD) is the most common form of dementia observed in western populations. A consistent histological feature of AD-affected brains is the extracellular accumulation of abnormal protein deposits mainly composed of aggregated  $A\beta$ . When autosomal dominant mutations in APP were shown to trigger early and aggressive forms of AD and when cell biology approaches indicated that these mutations systematically triggered modulations of  $A\beta$  production or yielded aggregation-prone  $A\beta$  species, the amyloid cascade hypothesis was proposed, in which  $A\beta$  is at the center of gravity [1-2].  $A\beta$  peptides are generated by sequential

processing of the transmembrane amyloid precursor protein (APP) by  $\beta$ - and  $\gamma$ -secretases [2]. First, APP is cleaved by  $\beta$ -secretase generating the membrane-bound fragment C99 (also referred to as  $\beta$ CTF), which then undergoes a secondary cleavage by  $\gamma$ -secretase leading to the release of soluble  $A\beta$  peptides. This sequence of proteolytic events is considered as the amyloidogenic pathway.  $\gamma$ -secretase is a membrane protein complex composed of Aph-1, Pen-2, nicastrin and presenilins (1 and 2) [3-5] and mutations in both *PSEN1* and more rarely *PSEN2* genes, encoding presenilin 1 and presenilin 2, respectively, are known to cause familial AD [6-7]. Transgenic mouse models harboring

mutated APP and/or mutated presenilins recapitulate present age-dependent extracellular amyloid deposits and other AD-related anatomical stigmata. Mounting data indicate that not only A $\beta$ , but also other catabolites of APP accumulate in the AD brain, and our previous work, based on the 3xTgAD (APP<sup>swe</sup>, PS1M106V, TauP301L) mouse model, indicated a particularly strong age-dependent intraneuronal accumulation of the C99 fragment, the direct precursor of A $\beta$  [8-9]. In the 3xTgAD mouse, C99 accumulation occurs in specific AD-related brain areas (hippocampus and cortex) and appears at very early stages much before any detectable A $\beta$ . Importantly, works from other groups later confirmed that early intraneuronal C99 accumulation occurs in several other mouse models, including the APP<sup>E693Q</sup> (Dutch mutation) expressing mouse [10], the TgCRND8 (Swedish and Indiana mutations) mouse [11] and the J20 mouse (Swedish and Indiana mutations) [12]. Thus, C99 accumulation appears as a common anatomical and biochemical denominator observed in AD mice models, could well be envisioned as an etiological trigger in AD pathology.

### **C99 accumulation is both a consequence and a cause of early lysosomal-autophagic dysfunction**

Our work showed that C99 mainly accumulates within endosomal, lysosomal and autophagic structures [8], which also correspond to the principal intracellular sites for amyloidogenic APP processing [13]. We found that C99 accumulation in the 3xTgAD mouse did not result from either increased  $\beta$ -secretase or reduced  $\gamma$ -secretase cleavages, but was rather due to early AD-associated lysosomal-autophagic impairment [8]. Thus, C99 accumulation was similar in both 3xTgAD and 2xTgAD mouse (APP<sup>swe</sup>, TauP301L), although the latter mice displayed little if any A $\beta$  as expected from the absence of mutated presenilin-1 [8]. In agreement with lysosomal dysfunction in both mice, C99 accumulated within abnormally large cathepsins- and lamp-positive structures, which number was also increased in C99-positive neurons [8-9]. Importantly, endolysosomal-lysosomal dysfunc

tion is also a key feature in human AD pathology and is consistently viewed as one of the earliest pathological events taking place in this disease. This dysfunction is reflected by the early appearance of morphological and functional abnormal endosomes and a progressive failure of lysosomal and autophagic degradation leading to the build-up of undigested autophagic vesicles [14]. Our data from mouse models indicated that C99 accumulation itself could contribute to this pathology. Indeed, the pharmacological inhibition of  $\gamma$ -secretase in young animals led to increased C99 levels (and C99-derived APP-CTFs, see below) but also to exacerbated lysosomal impairment [9]. Strikingly, the group of Dr Levy drew the same conclusions from a study based on a transgenic mouse model harboring the Dutch mutation, the APP<sup>E693Q</sup> mouse [10]. As in the 3xTgAD mouse, C99 accumulation was observed within enlarged LAMP-1, LAMP-2 and cathepsin D-positive structures and the number of these structures were strongly increased in C99-positive cells [9-10]. These findings showed that C99 could alter lysosomal function in addition to its earlier reported deleterious effects on endosomal dysfunction [14-16]. For instance, in fibroblasts from Down's syndrome, a cause of early-onset AD-like pathology due to an extra copy of APP, C99 was found to accumulate within early endosomes and cause both morphological and functional endosomal abnormalities, including up-regulated expression of endocytic proteins, aberrant endocytosis and impaired endosomal transport [15]. These deleterious effects were reversed in cells treated with a  $\beta$ -secretase inhibitor, but increased in the presence of a  $\gamma$ -secretase inhibitor, demonstrating that C99 but not A $\beta$  should underlie these abnormalities [15]. We and the group of Dr Levy also found that this intraneuronal accumulation is associated with microglial activation and astrogliosis [9-10]. These inflammatory responses were particularly important in  $\gamma$ -secretase inhibitor-treated mice (displaying little if any A $\beta$  and high APP-CTF levels) demonstrating that inflammation was indeed linked to C99 accumulation [9]. Again, the group of Dr Levy, working on the APP<sup>E693Q</sup> mouse, came to the same conclusion, since neuroinflammation in these mice appeared at

very early ages before any A $\beta$  detection [10]. These observations are in agreement with more general conclusions linking intraneuronal accumulation of biologically inactive aggregated proteins to neuroinflammatory activation [17].

**C99 accumulation exacerbates C99-derived C83 production and generate high molecular weight APP-CTFs-related species.**

Although, it was found that the major accumulating intraneuronal immunostaining corresponded to C99, our work also indicated that part of it could correspond to C83. This catabolite mainly arises from non-amyloidogenic processing of APP by  $\alpha$ -secretase, which occurs primarily on the plasma membrane. However, this apparently paradoxical observation could be explained by the susceptibility of C99 itself to proteolysis by  $\alpha$ -secretase [18-19] and suggests that  $\alpha$ -secretase activity should also be efficient within organelles of the endolysosomal system. Particularly high C99-derived C83 levels were found in animals treated with  $\gamma$ -secretase inhibitors, indicating that  $\gamma$ -secretase inhibition not only protected endogenous C83 from  $\gamma$ -secretase-mediated breakdown, but also somehow favored the cleavage of C99 by  $\alpha$ -secretase. Strikingly, this interplay between  $\gamma$ - and  $\alpha$ -secretase activities has already been described [20, 41]. Beside C99 and C83, expression analysis revealed the presence of high-molecular weight APP-CTFs in cells or brain tissue accumulating high levels of C99. We observed these APP-CTFs in cells expressing APP bearing the Swedish mutation, which favors the  $\beta$ -secretase cleavage of APP but also in cells expressing directly the C99 fragment, indicating that these APP-CTFs derived directly from C99 and did not correspond to other putative APP-derived metabolites of higher molecular weight. In this context, recent work has focused on the  $\eta$ -secretase-derived APP cleavage product, APP-CTF $\eta$  [21-22]. Indeed, this 30 kDa APP-CTF can be recognized by anti-APP C-terminal antibodies and it has a size close to one of the high-molecular weight APP-CTFs observed in our study. We hypothesized that this 30kDa fragment could correspond to homodimers of

C99, since several *in vitro* studies have shown that C99 can form homodimers [23-26]. This homodimerization is favored by three glycine-xxx-glycine (GxxxG) motifs in the juxtamembrane and transmembrane regions of C99 [23], but its influence on  $\gamma$ -secretase-mediated APP processing remains controversial. It was reported that C99 homodimerisation hampered  $\gamma$ -secretase cleavage thus lowering A $\beta$  production [26], while others reported on increased cleavage yielding enhanced A $\beta$  production [23]. Interestingly, it was also reported that cholesterol could bind to C99 and compete with it, leading to a reduced homodimerization and increased A $\beta$  production [25], in agreement with Winkler's team conclusion [26]. Beside the 30kDa APP-CTF, we also observed the presence of additional high molecular weight APP-CTFs. Although never reported so far, our findings suggested that these immunoractivities could correspond to homodimers of C99 or C83, heterodimers of C99 and C83, as well as heterodimers of other APP-CTFs and cholesterol bound APP-CTFs. Of note, these APP-CTFs were recognized *in situ* in our mouse models by the aggregate-specific antibodies NU1 and NU4 [27] indicating that these species could well harbor an "aggregate-like" conformation within the endolysosomal-autophagic vesicle membranes. Further immunological characterization of this intracellular staining revealed that these high molecular weight species were not recognized by APP C-terminal directed antibodies, suggesting that dimers formation could well induce a conformation change precluding their C-terminal epitope accessibility/interaction and antibody recognition. Immunostaining of brains of  $\gamma$ -secretase inhibitor-treated mice with NU1 increased intracellular staining, indicating that the label could not be accounted for by aggregated A $\beta$  [9]. Thus, our work not only revealed the early occurrence and accumulation of intraneuronal C99 monomers, but was also the first study to document the accumulation of C99-derived C83 and higher molecular weight APP-CTFS (dimers), *in situ* within the same intracellular structures. One could speculate that either alone or after

physical interaction, these species could cooperate to trigger neurotoxic phenotypes.

### **Brain network or synaptic alterations can be linked to early C99 accumulation**

Several lines of evidence highlighted hippocampal synaptic dysfunction as a cause of memory impairment in AD [28]. In animal models, this impaired synaptic function appears as an early event and leads to defects in memory much before the appearance of amyloid plaques and neuronal degeneration [29]. In particular, AD has been associated with defects in synaptic transmission, long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie the cellular mechanisms of learning behavior and memory consolidation. Mounting evidence suggest that these synaptic dysfunction defects are triggered by soluble A $\beta$  oligomers and much effort in recent years has been made to identify the receptors and signaling pathways implicated in oligomers-induced toxic effects [30-32]. Thus, A $\beta$  was found to bind to receptors for glutamate, nicotinic acetylcholine, cellular prion protein, neurotrophins (p75NTR) and ephrin among others [32]. In order to study the implication of C99 in synaptic alterations, we recently analyzed hippocampal LTP in C99-expressing mice (AAV-mediated C99 expression) [9]. Indeed, our results indicated that hippocampal LTP was significantly reduced in young C99-expressing mice, as compared to control mice infected with control virus. The inhibition of  $\gamma$ -secretase did not rescue LTP alterations indicating that C99 rather than A $\beta$  mediated these effects [9]. In agreement with these findings, two lines of independent observations also indicated that the hippocampal LTP alterations observed in the 3xTgAD mouse (at least at the early stages of the pathology) should likely be linked to C99. Firstly, the synaptic alterations were temporally and spatially correlated with C99 accumulation [33]. Secondly, these electrophysiological modifications were identical in the 2xTgAD (APP<sup>swe</sup>, TauP301L), which accumulate similar levels of C99, while A $\beta$  levels remain poorly detectable in this mouse model [34]. Moreover, it was found that spatial learning (using the Morris water maze test) was altered in both the 2xTgAD and 3xTgAD mice, as

compared to control mice, although with a higher impact in the 3xTgAD mice, indicating that both C99 and A $\beta$  could well contribute to these alterations [34]. Recently, it was proposed that memory impairment is not only explained by LTP/LTD defects, but is also associated with changes in brain circuits and network activities reflected by hyperexcitability and spontaneous epileptiform activity [35]. These alterations were also triggered by pathological A $\beta$  oligomers [35-36]. Nevertheless, a number of recent works demonstrated that network alterations appeared at very early stages much before A $\beta$  detection, proposing that other candidates could be responsible for these electrophysiological alterations. For instance, the group of Dr William observed alterations in hippocampal network oscillations and theta-gamma coupling in the subiculum of the hippocampus of the TgCRND8 AD mouse brain, at early "A $\beta$ -free" stages [37]. Later work from the same group demonstrated similar electrophysiological alterations in young J20 mice and these abnormalities were correlated with early C99 accumulation, rather than with A $\beta$  accumulation. Of importance, it was found that the treatment with a  $\beta$ -secretase inhibitor (that fully prevented C99 formation) rescued these alterations thus demonstrating that they should be directly linked to C99 [12].

### **Conclusion**

There is a still growing body of evidence suggesting a critical role of C99 in Alzheimer disease mouse model neuropathology. The possibility that C99 could contribute to AD etiology independently of A $\beta$  is supported by C99-induced endosome and lysosome dysfunction, neuroinflammation and electrophysiological alterations in various mice models at early stages of the pathology, at which little if any A $\beta$  is detectable. Very little is still known about the role of C99 in human pathology, since most researches have so far focused on A $\beta$  and have been performed at the end-stages of the disease, at which initial C99 toxicity may be overtaken by late A $\beta$  accumulation and toxicity. Nevertheless, several papers have reported that C99 levels are indeed significantly elevated in human AD brain tissues, as compared to control brains

[16, 38-39] indicating that C99 accumulation also occurs in human pathology and can be monitored also at late stage of the disease. Overall, one can envision an AD setting initiated by C99, while disease progression and late cognitive alterations should be attributed to C99, A $\beta$  and certainly other APP-derived catabolites [40].

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