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Soluble A β oligomer production and toxicity

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Abstract

For nearly 100 years following the first description of this neurological disorder by Dr. Alois Alzheimer, amyloid plaques and neurofibrillary tangles have been hypothesized to cause neuronal loss. With evidence that the extent of insoluble, deposited amyloid poorly correlated with cognitive impairment, research efforts focused on soluble forms of A β , also referred as A β oligomers. Following a decade of studies, soluble oligomeric forms of A β are now believed to induce the deleterious cascade(s) involved in the pathophysiology of Alzheimer's disease. In this review, we will discuss our current understanding about endogenous oligomeric A β production, their relative toxicity *in vivo* and *in vitro*, and explore the potential future directions needed for the field.

Keywords

amyloid-beta; oligomer; Alzheimer's disease; transgenic mouse

INTRODUCTION

With the vast majority of baby boomers now entering the dreaded 65 years of age mark associated with increased prevalence for Alzheimer's disease (AD), numerous occidental countries are facing a possible dantesque scenario for their respective health plans. More than 100 years following the initial publication of the description of its symptoms and neuropathology, and despite an accumulation of knowledge about the pathophysiology of AD, drugs capable of preventing, limiting and halting the disease are desperately needed. The major hurdle to the identification and development of new compounds targeting AD relies partly on the paradigm shift that occurred in the last 10–12 years: the emergence of amyloid- β (A β) oligomers as potential culprits of this brain disorder.

I. PRODUCTION OF OLIGOMERIC A β ASSEMBLIES

In AD, soluble A β monomers can form higher-order assemblies ranging from low-molecular weight oligomers (*e.g.* dimers and trimers) to mid range-molecular weight oligomers (*e.g.* A β *56) to protofibrils and fibrils (Figure 1). However, the exact relationship of these species to each other remains speculative, mostly because of their intrinsic nature as soluble

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globular intermediates. Despite this hurdle, multiple independent groups have observed apparent endogenous A β oligomers in protein expression studies using human *postmortem* brain tissue and using animal and cell lines genetically engineered to produce and secrete A β species.

A. Detection of soluble A β oligomers in early studies—In the first half of the 1990s, two studies revealed that fibrillar amyloid load poorly correlated with cognitive impairment (Terry *et al.* 1991, Dickson *et al.* 1995). In 1999, two additional reports indicated that soluble non-fibrillar A β levels correlated strongly with the severity of the disease (McLean *et al.* 1999) and synaptic loss (Lue *et al.* 1999), suggesting the possibility that soluble species of A β might play a larger role in AD pathophysiology than deposited amyloid. Particularly, McLean and coworkers found the presence of SDS-stable A β oligomers with an apparent molecular weight of ~8 and ~12 kDa in both soluble and insoluble fractions of AD brain tissue. Of note, apparent levels of A β dimers were more abundant in the insoluble fraction than those in the soluble fraction. In parallel to these *in vivo* studies, evidence emerged that cell lines overexpressing mutant forms of APP linked to familial AD were able to produce A β assemblies intracellularly and secrete soluble A β dimers and trimers (Podlisny *et al.* 1995, Podlisny *et al.* 1998, Walsh *et al.* 2000). In the latter study, the Chinese hamster ovary cell line 7PA2, stably overexpressing APP-V717F, was compared to human primary cortical neurons. Following immunoprecipitation with either 40- or 42-end specific antibodies (2G3 and 21F12), only monomeric A β was detected in the conditioned medium of human cells compared to monomers, dimers and trimers identified in 7PA2 (Walsh *et al.* 2000). Similar findings were found for cellular lysates even though the results need to be interpreted with caution, as carboxyl-terminus fragments of APP were not immunodepleted out of the protein extracts used.

Not only did these observations suggest that neurons might produce a different profile of A β oligomers than the genetically engineered line 7PA2, but they also demonstrated that A β oligomerization might be a natural process *in situ*.

B. Current understanding of oligomeric A β production—Following these original studies, soluble A β molecules became the specific focus of subsequent studies in attempt to shed light on their possible contribution to AD pathophysiology. In order to try to clarify the fast accumulating data on this topic, we classified these reports based on the putative A β oligomers directly involved. These endogenous assemblies included dimers, trimers, A β *56, species immunoreactive to ADDLs/globulomers antibodies, and annular protofibrils.

1. Dimers: A β dimers are probably the most studied oligomeric species at the present time. Multiple reasons account for the latter: (1) A β dimers are SDS-stable (McLean *et al.* 1999), (2) A β dimers brain levels are elevated in AD mouse models and in subjects with AD (Kawarabayashi *et al.* 2004, Shankar *et al.* 2008, Shankar *et al.* 2009, Mc Donald *et al.* 2010), (3) size-exclusion chromatography segregated A β dimers from AD brain extracts impair long-term potentiation (Shankar *et al.* 2008). While the potency of A β dimers appears to be remarkable (Reed *et al.* 2009), the question relative to the origin of dimers remains nevertheless speculative.

In animal models of AD such as Tg2576 (Kawarabayashi *et al.* 2004) and J20 (Shankar *et al.* 2009, Meilandt *et al.* 2009), A β dimers can be found in soluble protein extracts at 10–12 and 10–14 months of age, respectively. Interestingly, these ages correspond to amyloid plaques depositing in cortical areas (Mucke *et al.* 2000, Kawarabayashi *et al.* 2001), suggesting that A β dimers and fibrillar A β are related to each other. Figure 2 illustrates that the relative abundance of A β dimers and monomers detected in the formic-acid soluble fraction (containing fibrillar proteins) sharply increased starting at 12 months of age in Tg2576.

Specifically, FA-soluble A β monomers followed a sigmoidal profile similar to the one previously described (Kawarabayashi et al. 2001). In contrast, dimeric A β levels showed an exponential profile in Tg2576 (Figure 2B). Since A β monomers reached a plateau prior than A β dimers in this model (Figure 2B, C), it is tempting to hypothesize that, dimeric A β levels appear to follow plaque accumulation *in vivo* in this model (Kawarabayashi et al. 2001). In plaque-free brains of young transgenic mice, A β dimers have yet to be found. Earlier work (Kawarabayashi et al. 2004) reported the presence of A β dimers in lipid raft preparations from 6-month-old Tg2576, but later studies revealed that specific lipid enrichment could trigger A β oligomerization artificially (Yu et al. 2005, Kim et al. 2006), questioning the intrinsic existence of these species in very young Tg2576 mice.

Similarly in human studies, apparent endogenous A β dimers were only found in brains of subjects with AD (Shankar et al. 2008, Mc Donald et al. 2010) when comparing individuals with extensive amyloid and tau pathology and subjects devoid of amyloid accumulation. In the former study (Shankar et al. 2008), proteins were extracted from subjects without AD (mean age = 65.6 $\pm$ 12.1 years; n = 3) or with severe AD (mean age = 78.1 $\pm$ 5.8 years; n = 7). A β dimers were identified only in the AD group, even though these groups were not normalized for age or amyloid burden. Thus, A β dimers appear to be closely linked to fibrillar amyloid in humans as well.

While these *in vivo* studies suggest that dimeric A β and plaques may play a deleterious and mysterious tango in the brain, the origin of dimers still remains elusive. Previous research revealed that primary cortical neurons from Tg2576 produce monomers and trimers; A β dimers were not detected under the experimental conditions used (Lesne et al. 2006) indicating that neurons preferentially produce and secrete monomers and trimers but not dimers. More recently, our own group observed that A β dimers are in fact produced and secreted by primary neurons but at very low amounts (1:20 and 1:15 ratio to monomers and trimers respectively; Figure 3A). These results are in sharp contrast to the relative abundance of dimers in the conditioned medium of 7PA2 cells (Podlisny et al. 1998, Walsh et al. 2002). The reason why dimers are produced and secreted so prominently in this cell line remains unknown, but the profile observed by biochemical analyses performed by our own group clearly differs from those from primary neurons (Figure 3B). Lastly, dimers are found as what appears to be the primary oligomeric species formed in mice overexpressing the E693 Δ APP mutant (Tomiya et al. 2010). Identified in 2008, this deletion within the mid-region of A β engages A β peptides to form preferentially oligomeric species instead of forming fibrils (Tomiya et al. 2008, Tomiya et al. 2010). Of interest, these A β dimers are accumulating intracellularly and co-segregated within the insoluble protein fraction extracted with formic acid but not within soluble protein fractions (Tomiya et al. 2010). These independent findings further support the hypothesis that endogenous A β dimers are the molecular brick for fibrillar A β *in vivo*. Identifying what might favor dimeric A β formation in absence of mutation may shed light on the origin of these assemblies.

2. Trimers: Several lines of evidence indicate that A β trimers might constitute the molecular brick for non-fibrillar assemblies of A β . First, as mentioned in the above section, trimers are the preferential, most abundant species produced and secreted by primary neurons *in vitro* (Lesne et al. 2006), even though the origin of trimeric A β remains elusive. It is possible that this preference might be due to lower energetic requirement to form trimers or due to the absence of a chaperone protein X allowing the conversion to dimers. Second, trimers are present in brain tissue of Tg2576 as early as embryonic day 14 and its expression persists throughout life (Lesne et al. 2006). Analyzing extracellular- and intracellular-enriched protein fractions in these mice indicated that brain levels of A β trimers steadily but very slowly rise with aging (Lesne et al. 2006). These findings were mirrored in human studies where trimers are found as early as 1 year of age, contrasting with brain A β dimers

which were not detected in subjects until 50–60 years of age in TBS extracts (Lesne *et al.* unpublished data).

Importantly, when comparing Tg5469 and Tg2576 lines, which express equal amounts of human APP but vastly different levels of A β due to the presence of the Swedish mutation in the latter line, A β trimers could not be readily observed in Tg5469 brains (Ma *et al.* 2007). This suggests that the formation of trimers *in vivo* appears to be dependent on the levels of A β production. Cumulatively, these findings suggest that trimers are the earliest endogenous oligomeric A β species produced and secreted by neurons.

3. A β *56: Based on the finding that neurons preferentially secrete A β trimers, it was likely that these assemblies could form larger oligomeric species based on multiples of 3, *i.e.* hexamers (6-), nonamers (9-) and dodecamers (12-), in absence of deposited A β . Examining young Tg2576 mice prior to plaque formation, apparent trimers (13 kDa), hexamers (27 kDa), nonamers (40 kDa) and dodecamers (56 kDa) were detected using antibodies targeting the N-terminus and central domain of A β , such as 6E10, and using the A11 antibody detecting prefibrillar oligomeric proteins (Lesne *et al.* 2006, Kaye *et al.* 2003). The discovery of A β *56, a putative dodecameric A β assembly, derived from the observation that cognitive decline in Tg2576 starts at 6 months of age, when A β *56 first appears, and remains relatively stable for months (up to 15-months of age) when numerous endogenous A β species (soluble monomers, insoluble monomers, dimers, trimers) accumulate in brain tissues many fold (Lesne *et al.* 2006). A β *56 can be detected with multiple antibodies, which contribute to establishing the antibody reactivity profile of this A β species: A11+, OC-, 6E10+, 4G8+, A β _{x-40}+, A β _{x-42}+, 82E1-. This epitope specificity allows to categorize A β *56 as a non-fibrillar A β oligomer (Figure 4).

Importantly, the presence of A β *56 was also reported in other APP transgenic mouse models of AD, *e.g.* J20 (Cheng *et al.* 2007, Meilandt *et al.* 2009), Arc6/48 (Cheng *et al.* 2007), 3xTgAD (Oddo *et al.* 2006) and APP23 (unpublished data), clearly arguing against the possibility that it is an artifact intrinsic of one isolated mouse model.

Contrary to dimers and trimers, which can be detected *in vitro*, A β *56 is not detected in cellular protein extracts of cultured neurons or in conditioned media indicating that it is not produced and secreted by primary neurons (Lesne *et al.* 2006). This finding suggests that this assembly requires a co-factor X, present in brain tissue, to promote its formation. Furthermore, aging must regulate the expression of this unidentified co-factor X in order to explain the sudden accumulation of A β *56 in 6-month-old Tg2576 brains.

In mouse models, A β *56 is present in two soluble protein fractions, extracellular-enriched (Lesne *et al.* 2006, Lesne *et al.* 2008) and membrane-enriched extracts (Cheng *et al.* 2007), indicating that this assembly is formed extracellularly and may interact with surface receptors at neuronal membranes. Similar segregation was observed in studies with a large human cohort ($n = 135$; (Lesne *et al.* unpublished data)). When comparing across clinical diagnosis groups, A β *56 brain levels seem to peak during the preclinical stage of AD and decrease in MCI and AD subjects (Lesne *et al.* unpublished data). The data are in agreement with evidence gathered in Tg2576 suggesting that A β *56 may form in the aging brain prior to the formation of amyloid plaques.

Of interest, the relative levels of soluble A β *56 in the extracellular protein fraction of human brain tissue showed no relationship with A β dimers (Figure 5A). In contrast, brain levels of human A β *56 were positively correlated to trimeric soluble A β levels in the same fraction (Figure 5B). This observation further supports our hypothesis that trimeric A β might constitute the building block for larger oligomers in the non-fibrillar pathway.

4. ADDLs and Globulomers: Even though this review aims at discussing accumulated findings about endogenous oligomeric A β species, tools initially developed to target synthetic soluble A β molecules (*e.g.* A β derived diffusible ligands or ADDLs and globulomers) appear to detect endogenous A β entities, even though the exact nature of these molecules needs further studying (Gong *et al.* 2003, Georganopoulou *et al.* 2005, Barghorn *et al.* 2005, Hillen *et al.* 2010). The antibodies termed NU-x and anti-globulomer antibodies (5598, 8F5, A-887755), developed by Klein's group at Northwestern and Abbott respectively, seem able to identify 35–60kDa oligomeric A β species recognized by these groups as dodecamers (Barghorn *et al.* 2005, Lambert *et al.* 2007, Hillen *et al.* 2010).

In Tg2576, ADDLs are increased ~5- to ~100-fold (Chang *et al.* 2003) at ages during which spatial reference memory remains unchanged. In addition, measurements of ADDLs in brain tissue and in CSF revealed 70-fold increases in AD compared to age-matched controls (Gong *et al.* 2003, Georganopoulou *et al.* 2005). These findings suggest that ADDLs levels rise sharply in AD, reminiscent of the marked elevation in A β dimers (Shankar *et al.* 2008).

Similarly to ADDLs, the use of mid-range molecular weight globular synthetic A β oligomers migrating at 38–48 kDa, termed globulomers, generated antibodies targeting alleged endogenous A β species. Due the apparent size of the synthetic assemblies detected, Barghorn and coworkers indicated that globulomers corresponded to A β dodecamers. In animal studies performed with Tg2576 mice, globulomers were mostly unchanged between 2.5 and 10 months of age prior to plaque formation (26%), but brain levels rose 496% at 12 months of age when amyloid plaques start forming (Kawarabayashi *et al.* 2001). Furthermore, immunohistochemical analyses using the anti-globulomer 8F5 antibody labeled amyloid plaques in brains of Tg2576 animals and subjects with AD (Barghorn *et al.* 2005). These findings indicate that, regardless of the exact endogenous A β species detected by anti-globulomer antibodies, these molecules are highly associated with amyloid plaques and are elevated in AD.

In both cases of ADDLs and globulomers, there has been some confusion that these “dodecamers” correspond to the endogenous 56 kDa A β assembly, A β *56 (Wilcox *et al.* 2011). However, several lines of evidence indicate that A β *56 and ADDLs/globulomers are different entities. First, A β *56 brain levels are remarkably stable between 6 and 15 months of age in extracellular-enriched fractions of Tg2576 (Lesné *et al.* 2006) while ADDLs and globulomers are vastly increased during that same time interval. Second, A β *56 levels in both extracellular and membrane-enriched protein fractions are lowered in AD compared to non-impaired age-matched controls (Lesné *et al.* unpublished data) while ADDLs/globulomers increase in AD (Gong *et al.* 2003, Georganopoulou *et al.* 2005, Barghorn *et al.* 2005, Nimmrich *et al.* 2008). Based on these observations, ADDLs antibodies likely detect *in vivo* species others than trimers and A β *56, possibly fibrillar oligomers or annular protofibrils (Glabe 2008). This subclass of oligomers is characterized by being immunopositive to the antibody OC (OC+) and immunonegative to A11 (A11–). Even though it is not known whether ADDLs and globulomers are OC+/A11–, the fact that ADDLs and globulomer antibodies detect amyloid plaques suggests that ADDLs correspond to fibrillar oligomers.

In addition to the point that ADDLs/globulomers and A β *56 represent different entities, the origin(s) of the endogenous A β species detected by ADDLs/globulomer antibodies is completely unknown. Further studies are needed to determine how these possible A β assemblies are produced and when they do form in the continuum aging-AD.

5. Annular protofibrils (~100–150kDa): Annular protofibrils (APFs) are pore-like structures that are believed to derive from the circularization of non-fibrillar A β assemblies

(Kayed *et al.* 2009). With a molecular size over 90 kDa, they resemble bacterial pore-forming toxins. APFs have been proposed to permeabilize lipidic membranes and induce cell death by altering neuronal homeostasis (Glabe & Kaye 2006, Lashuel & Lansbury 2006). Recent data revealed that APFs might constitute a distinct A β assembly from non-fibrillar oligomers (Kayed *et al.* 2009). Based on this work, APFs originate from pre-existing non-fibrillar oligomers, which upon some conformational change allows the recruitment of additional oligomers to form APFs. In 17–20 month-old APP23 mice, ~2% of examined synapses showed an accumulation of APFs using a specific α APF antibody (Kokubo *et al.* 2009). On a follow-up study, these same groups recently demonstrated that APFs are associated with diffuse plaques and intracellular punctate deposits in AD brain sections (Lasagna-Reeves *et al.* 2011). Despite very small specimen numbers ($n = 2/\text{group}$; $N = 4$), these findings indicate that APF exist *in vivo*.

Even though these new studies and conformation-dependent antibodies are allowing us to discriminate among the multiple oligomeric forms of A β *in vitro* and *in vivo*, we know nothing about the age at which APFs first form in animal models and in human brain tissue and CSF.

C. Future directions—With the emergence of endogenous oligomeric forms of A β , perhaps more than anticipated, our understanding of the conditions necessary to prepare synthetic A β oligomers and novel detecting tools (*e.g.* conformation-dependent antibodies such as A11 and OC) still needs improvement. Great confusion has risen in our field when comparing specific oligomers, particularly regarding *in vitro* and *in vivo* A β assemblies. If the working hypothesis is that A β oligomers are the real culprits in AD, it is therefore imperative that we collectively determine the origins of endogenous soluble A β species, when their respective levels are modified in transgenic models and human brain tissue across ages and finally in longitudinal studies using CSF. A longitudinal model extrapolated from our work in human cohorts (Lesné *et al.* unpublished data) is shown in Figure 6. In the model proposed, lower energy requirements for A β to form high molecular weight oligomers compared to lower molecular weight species would support the concept that A β *56 peaks earlier than A β trimers and dimers. If correct, this phenomenon would lead these high- n molecules to reach a plateau first, then allowing the accumulation of the low- n A β oligomers, otherwise used for oligomerization under non-saturating conditions. Such scenario would therefore lead to the accumulation of larger species first, followed by a second accumulation of lower assemblies. Further studies will be needed to determine whether this proposed model is correct.

Finally, the generation of novel, specific detection and diagnostic means is therefore a crucial avenue. With the aging of the population becoming a central concern for our respective health care systems, together, the field must find common grounds to foster collaborative projects and fasten our relatively speculative understanding of how endogenous A β oligomers are produced, when they appear and what/when they are modulated across preclinical subjects, patients with mild cognitive impairment or with AD.

II. TOXICITY OF OLIGOMERIC A β ASSEMBLIES

As described above, multiple forms of A β oligomers appear to exist and co-exist in brain tissue. While the field is still searching for avenues to identify the nature of the relationships between these various assemblies, we paradoxically know more about the deleterious effects of oligomeric A β than how they are produced *in vivo*.

A. Current knowledge about soluble A β oligomers-induced toxicity

1. Low molecular weight A β oligomers: dimers and trimers: Following the identification of SDS-stable A β oligomers in 7PA2 cells, it was important to determine whether these A β species were biologically active in order to set the stage for A β oligomers as potential toxins in AD. Conditioned medium of 7PA2 and CHO control cells were injected in rats and hippocampal long-term potentiation (LTP) was assessed. *In vivo* LTP was blocked upon injection of the medium containing A β oligomers (Walsh et al. 2002). After removal of A β species, the same medium had no effects on LTP indicating that soluble A β oligomers formed in 7PA2 cells inhibit neuronal function. To demonstrate that oligomers and not monomeric A β triggered this effect, 7PA2-derived species were injected into size-exclusion chromatography (SEC) columns. Fractions enriched for dimers/trimers inhibited LTP and cognition but not monomers (Walsh et al. 2005, Cleary et al. 2005).

To test whether 7PA2-derived A β dimers and trimers were directly related to synaptic loss, 7PA2 CM was applied to hippocampal organotypic cultures. Dendritic spine loss was observed after 3–5 days of exposure with these low molecular weight oligomers using a mechanism dependent on N-Methyl-D-Aspartate (NMDA) receptors (NMDAR) and calcineurin (Shankar et al. 2007). Despite these convincing findings, the nature of the soluble pathogenic A β species in human brain tissue or CSF was still speculative. To this end, soluble human brain tissue extracts from individuals with AD were obtained and segregated by SEC. Application of the total extract from AD brains blocked LTP (Shankar et al. 2008), reproducing the findings obtained with 7PA2 CM (Walsh et al. 2002, Walsh et al. 2005). In addition, the dimer-containing fraction inhibited LTP (Shankar et al. 2008) and facilitated LTD (Li et al. 2009) indicating that A β dimers alone can alter neuronal plasticity.

While attempting to elucidate the mechanism by which dimeric A β inhibits LTP, Li and colleagues demonstrated that NMDAR subunit 2B (NR2B or GluN2B)-containing receptors were indirectly necessary for LTP inhibition triggered by dimers. Briefly, dimers alter glutamate reuptake at synapses leading the accumulation of extracellular glutamate and excessive stimulation of extrasynaptic receptors (Li et al. 2009). As a result of activation of these receptors *in vitro*, the calpain/p38 axis is activated and the survival promoting ERK1/2 pathway is inhibited within 6 hours of exposure with 7PA2-derived A β dimers/trimers (Li et al. 2011).

2. Dimers: SEC-segregated A β dimers from AD cerebral cortex also induced tau-dependent cytoskeletal microtubule abnormalities in primary neurons (Jin et al. 2011). Treatment with dimers triggered tau hyperphosphorylation at disease-relevant epitopes (Jin et al. 2011). Altogether, these results demonstrate that endogenous A β dimers are synaptotoxic. Kawarabayashi and colleagues reported that A β dimers could be detected in lipid raft isolations of 6-month-old Tg2576 mice. These authors suggested that the appearance of dimers coincided with the onset of memory decline observed in this APP transgenic line (Kawarabayashi et al. 2004). However, while brain levels of A β dimers increased extraordinarily (~500 fold), memory function did not further worsen arguing against the implied relationship between dimers and the onset of cognitive decline in this mouse model. Ultimately, the mid-size molecular weight A β oligomer A β *56 was related to initiating this memory impairment (Lesné et al. 2006). Even though A β dimers may not be responsible for the initial decline observed in 6-month-old Tg2576 animals, it is tempting to hypothesize that the second decline occurring between 13 and 15 months of age is due to the accumulation of A β dimers, considering the association between amyloid plaques and dimers. In support of this postulate, a recent study proposed that A β dimers rapidly form stable protofibrils to mediate synaptotoxicity (O'Nuallain et al. 2010).

3. Trimers: Contrary to A β dimers, very few studies have been conducted with naturally formed A β trimers (Townsend *et al.* 2006, Reed *et al.* 2009). Applying SEC-separated A β trimers from 7PA2 cells onto hippocampal slices led to greater inhibition of LTP than any low-*n* oligomers derived from 7PA2 CM, including dimers (Townsend *et al.* 2006).

Reed and coworkers (2009) compared the effects of various A β assemblies on cognitive function in the alternating lever cyclic ratio (ALCR) behavioral paradigm (Cleary *et al.* 2005). Among the species tested were SEC-fractionated dimers and trimers from 7PA2, along with trimers and A β *56 from Tg2576 brain tissue. Both trimers altered cognitive function regardless of the source (cell line or mouse brain), even though their relative potency was quite weak compared to that of dimers or A β *56. We do not know yet whether the molecular mechanism(s) mediating these effects is the same identified for dimers but these results indicate that trimers are capable of impairing neuronal function. Additional indirect evidence supports a deleterious role of A β trimers *in vivo*. While young Tg2576 do not appear to be impaired in the Morris water maze compared to non-transgenic animals at ages where A β trimers are the only species detected (Lesne *et al.* 2006), this observation does not take into consideration the potential beneficial effect of soluble APP- α (sAPP α) resulting from human APP overexpression. When compared to Tg5469 mice, which overexpress APP at similar levels (Ma *et al.* 2007, Westerman *et al.* 2002), Tg2576 mice performance in the Morris water maze was much lower than Tg5469 transgenic animals, suggesting that trimers are already impairing cognition in Tg2576 prior to the formation of A β *56 occurring at 6 months of age. Also, using APP transgenic rats, intraneuronal trimers were found to be the only oligomeric A β species present at 3 months of age, when a cognitive deficit was detected (Leon *et al.* 2010).

4. A β *56: Since A β *56 is only formed at low levels in the aging brain, studying its effects following purification represents a real technical challenge. Nevertheless, following affinity purification combined with size-exclusion chromatography, purified A β *56 caused transient memory impairment in young healthy rats demonstrating that this A β assembly impairs brain function *in vivo* (Lesne *et al.* 2006). First identified in Tg2576 mice, the brain expression of A β *56 was also found associated with memory decline in other APP transgenic mice modeling AD, namely J20, Arc48 and 3xTgAD (Oddo *et al.* 2006, Cheng *et al.* 2007). In human studies (*n* = 88), we found that A β *56 was positively correlated with soluble hyperphosphorylated tau at disease-relevant epitopes in AD brains (Figure 7). Additional analyses linking A β *56 and tau in the aging human brain are currently awaiting peer-review (Lesne *et al.* unpublished data) and the exact molecular mechanism by which A β *56 induces tau changes is under investigation.

A β *56 is not toxic *per se* as there is no neuronal cell loss in Tg2576 at the age of A β *56 formation. We hypothesize that A β *56 is able to perturb neuronal physiology without triggering cell death. This postulate could explain why this A β assembly impairs cognition in middle-aged Tg2576 and in young rats as well as the reversibility of its effect following acute injection (Lesne *et al.* 2006).

5. ADDLs/globulomer immunoreactive species: The toxicity of synthetic A β oligomers referred as ADDLs have been reviewed recently (Wilcox *et al.* 2011) and we will therefore not extend this discussion much further than the points addressed there. Specifically, we want to include recent observations that application of ADDLs on primary neurons led to the abnormal localization of tau to dendrites (Zempel *et al.* 2010). At the same time, Ittner and coworkers elegantly demonstrated that tau could be missorted to dendrites under pathological conditions (Ittner *et al.* 2010, Ittner & Gotz 2011). This effect is mediated by the tyrosine Src kinase Fyn, which has been associated with soluble A β toxicity since the late 1990s (Lambert *et al.* 1998). Importantly, when APP23 mice were crossed with mice

expressing a tau construct preventing Fyn-mediated tau relocalization to dendrites, neuronal/cognitive deficits induced by A β were blocked (Ittner *et al.* 2010). The activation of Fyn induced by ADDLs appears to be physiologically relevant as several *in vivo* studies demonstrated a crucial involvement of Fyn in A β -induced neuronal/cognitive dysfunction (Chin *et al.* 2004, Chin *et al.* 2005, Roberson *et al.* 2011). Moreover, Fyn can hyperphosphorylate tau at tyrosine 18 (Y18) which accumulates in brains of AD subjects and of tau-overexpressing mice (Lee *et al.* 1998, Lee *et al.* 2004, Bhaskar *et al.* 2005, Bhaskar *et al.* 2010). Based on the latter, genetic ablation of tau prevented cognitive impairment triggered by A β /Fyn (Roberson *et al.* 2011). Thus the triad A β /Fyn/tau seems to play an important role in A β -induced deficits related to AD (Haass & Mandelkow 2010, Ittner & Gotz 2011), even though the exact A β species and the molecular mechanism linking A β oligomers to Fyn is unknown. The status of pY18-tau also needs to be examined in the transgenic mouse lines used by Roberson and coworkers in order to validate whether A β modulates tau via Fyn signaling.

In contrast to A β molecules detected by ADDL antibodies, A β species immunoreactive for globulomer antibodies impair mitochondrial function (Eckert *et al.* 2008) and inhibit presynaptic P/Q-type calcium channels (Nimmrich *et al.* 2008). Based on the latter work, these assemblies appear to attack the presynaptic end of the synapse as opposed to the postsynaptic terminal for A β dimers and ADDL-positive entities.

6. Annular protofibrils: The existence of annular protofibrils (APFs) *in vivo* has only been recently confirmed using a novel antibody, α APF, specifically designed to target this A β assembly (Kayed *et al.* 2009, Kokubo *et al.* 2009, Lasagna-Reeves *et al.* 2011). It is however unclear whether purified endogenous APFs display the same neurotoxic properties as their synthetic counterparts (Kayed *et al.* 2009). This has not been the case for dimers for instance, with A β S26C dimers and endogenous A β dimers displaying remarkable potency differences (Jin *et al.* 2011).

B. Proposed surface mediators of endogenous A β oligomers—Whereas endogenous oligomeric A β species have been linked to cognitive and neuronal dysfunction, findings related to the molecular mechanisms by which these A β assemblies display their effects are only starting to emerge (Figure 8).

Based on the observations that A β impairs synaptic plasticity and that NMDAR are crucial regulators of LTP and LTD, soluble A β exposure was shown to reduce surface NMDAR expression (Snyder *et al.* 2005). This internalization of NMDAR resulted from the activation of α 7-nicotinic acetylcholine receptors (α 7-nAChR) and downstream effectors calcineurin (formerly known as protein phosphatase 2B) and striatal-enriched phosphatase STEP. The observed cascade of events briefly reported here suggested that certain oligomeric A β species present in the conditioned medium of APP-overexpressing N2A cells are capable of binding to the α 7-nAChR, ultimately leading to synaptic plasticity impairments. Unfortunately, the nature of the A β molecules present in the material used was not disclosed (Snyder *et al.* 2005) making it difficult to place these results in a larger context when comparing the effects of specific oligomers.

Although some studies have shown A β colocalized with NMDA receptors (NMDAR) at synapses (Lacor *et al.* 2007, Dewachter *et al.* 2009), it remains unclear whether this is a direct target of the peptide. Several molecular targets for synthetic oligomeric A β at the neuronal surface have been proposed including the receptor for advanced glycation end products (RAGE) (Sturchler *et al.* 2008), metabotropic glutamate receptors mGluR5 (Renner *et al.* 2010), PrP^c (Lauren *et al.* 2009), EphB2 (Cisse *et al.* 2011a). Among them, two receptors particularly stimulate our interest: PrP^c and EphB2.

A β was first shown to interact with RAGE in the mid 1990s (Yan *et al.* 1996) and was subsequently proposed to regulate A β accumulation in the brain (Deane *et al.* 2003). While RAGE was identified as potential binding receptor for oligomeric A β species prior to the other protein receptors, its discovery as mediator of oA β was obtained using high concentrations (10 μ M) of synthetic A β _{1–40} (Sturchler *et al.* 2008). Using 10 μ M mixtures of oA β with unknown stoichiometry or relative abundance, RAGE was found to bind oA β when compared to A11-immunonegative A β fibrils or aggregates (Sturchler *et al.* 2008). It is unfortunate that monomeric A β preparations were not tested in parallel to synthetic oA β . In addition, deletion of RAGE rescued LTP inhibition triggered by 200 nM of synthetic oA β (Origlia *et al.* 2008). The protein kinase p38 was proposed to serve as a downstream effector of the RAGE pathway activated by oA β in this paradigm (Origlia *et al.* 2008). It is however still unknown whether endogenous soluble A β oligomers do co-immunoprecipitate with RAGE in brains of AD patients or of transgenic mouse models of AD. Similarly, we do not know whether more physiological concentrations of oligomeric A β , *i.e.* low nM based on previous reports (Walsh *et al.* 2002, Shankar *et al.* 2008) would activate signaling pathways downstream of RAGE. In animal models, while overexpression of RAGE or a dominant negative form of RAGE in mice overexpressing the *Swedish/Indiana* mutant of APP enhanced or decreased behavioral deficits respectively (Arancio *et al.* 2004), gene ablation of RAGE in transgenic mice overexpressing the *Artic* mutant of APP did not rescue cognitive impairment (Vodopivec *et al.* 2009). Altogether, more evidence is needed to rigorously claim that endogenous soluble A β oligomers bind and activate RAGE.

Over the past few years, the prion protein has probably been the most controversial receptor for A β oligomers (Balducci *et al.* 2010, Kessels *et al.* 2010, Calella *et al.* 2010, Benilova & De Strooper 2010, Barry *et al.* 2011, Freir *et al.* 2011, Cisse *et al.* 2011b). The cellular form of the prion protein PrP^c has been identified in a high-throughput screen using non-neuronal cell lines and synthetic preparations of A β (Lauren *et al.* 2009). Based on this screen, very large synthetic A β oligomers (~500 kDa with an estimated number of A β molecules ranging between 50 < *n* < 100) were found to bind to the 95–105 region of PrP^c. When applied to primary neurons, these A β assemblies interacted with plasma membranes in a fashion reminiscent from the binding seen for ADDLs (Lacor *et al.* 2004). Genetic deletion of PrP^c partly reduced this apparent binding to cells indicating that PrP^c might act as one of multiple receptors for synthetic A β oligomers (Lauren *et al.* 2009). Controversy has existed with regards to the alleged requirement of PrP^c expression for synthetic A β to inhibit LTP (Lauren *et al.* 2009, Kessels *et al.* 2010, Calella *et al.* 2010, Barry *et al.* 2011, Freir *et al.* 2011). One possible explanation for the lack of reproducibility of these findings between *in vitro* and *in vivo* models may relate to the heterogeneity of synthetic oligomeric A β mixtures used and to a different relative abundance of the A β assemblies across systems. Another might relate to the fact that the effect of PrP was only tested in young APP transgenic mouse models (Calella *et al.*, 2010; Cisse *et al.*, 2011). Considering that endogenous brain A β oligomers are produced at different ages, it is therefore conceivable that PrP^c could mediate oA β -induced neuronal dysfunction in older animals. To resolve part of this issue, Dr. Walsh's and Dr. Rowan's groups took advantage of an epitope masking PrP^c antibody (D13, targeting the 96–104 region of PrP) to prevent A β from interacting with PrP^c (Barry *et al.* 2011). When soluble A β -containing extracts of AD brain were injected intracerebroventricularly, LTP was inhibited as previously reported (Shankar *et al.* 2008). Pre-injection of D13 completely abolished, oligomeric A β -induced inhibition of LTP, indicating that endogenous A β oligomers can interact with PrP^c to alter LTP. Finally, these experiments were repeated in PrP-null mice in which LTP deficits induced by A β -containing extracts of AD brain were abolished indicating that PrP mediates the neurotoxic effects of certain A β species (Freir *et al.* 2011). More studies are however needed to directly show which endogenous A β oligomer mediates this effect, where they colocalize and what is the cellular pathway linking A β , PrP^c and neuronal dysfunction. The Ephrin B2 receptor EphB2

is enriched at synapses where it clusters with NMDA receptors (Dalva *et al.* 2000) and it has also been proposed to act as a receptor for endogenous A β oligomers (Cisse *et al.* 2011a). EphB2 expression was found decreased in human AD hippocampal extracts (Simon *et al.* 2009) and ADDL application to mature hippocampal neurons lowered EphB2 expression by 60% (Lacor *et al.* 2007). When using biotinylated synthetic A β oligomers, trimers co-immunoprecipitated with EphB2 in immature cortical and hippocampal neurons (DIV7); when 7PA2 CM was applied to neurons, dimers and trimers were pulled down with anti-EphB2 antibodies (Cisse *et al.* 2011a). This apparent discrepancy further warrants caution when designing experiments with synthetic intermediate assemblies. Even though these data indicate that EphB2 may bind oligomers in *in vitro* paradigms and that EphB2 is an important player in A β -induced neuronal dysfunction, it could have been more medically relevant to examine whether similar co-immunoprecipitations can be done using human and APP transgenic brain tissues, and specifically identify the endogenous A β species binding to EphB2 *in situ*.

C. Toxicity and relative concentration of oligomeric A β molecules—While intensive efforts are invested in trying to identify the potential mediators of A β oligomers at the neuronal plasma membrane, another “ghost from the past” parameter impedes the progress of our understanding of oA β -induced toxicity: the relative concentration of exogenously applied A β oligomers. Already in the 1990s, the question of how much A β to use in experimental settings was questioned. Nowadays, this conundrum still holds true for A β oligomers, as recently illustrated (Puzzo *et al.* 2008). In a seminal article, low nanomolar (nM) concentrations of 7PA2-derived A β oligomers were only needed to disrupt LTP (Walsh *et al.* 2002). Of note, the relative concentration of A β oligomers was determined by comparison with monomeric A β integrating monomeric A β enzyme-linked immunosorbent assay (ELISA) with SDS-PAGE/densitometry analyses. Using relative concentrations within the low nM range (0.5–10 nM), exogenously applied A β oligomers were found capable of inhibiting cognition (Cleary *et al.* 2005, Reed *et al.* 2009), triggering dendritic spine loss (Shankar *et al.* 2007, Shankar *et al.* 2008), and tau hyperphosphorylation (Jin *et al.* 2011). In contrast, synthetic oligomeric A β preparations are traditionally used at much higher concentrations ranging from 0.1–20 μ M (Lacor *et al.* 2004, Deshpande *et al.* 2006, Lacor *et al.* 2007, Shankar *et al.* 2008, Renner *et al.* 2010, O’Nuallain *et al.* 2010). The apparent difference in relative concentration of A β could therefore explain the discrepancies in the potential mechanisms mediating oligomeric A β toxicity.

D. Future directions—Our understanding of the deleterious events triggered by oligomeric A β species has greatly improved in the past 3–4 years but much more is needed before we can establish an integrated view on this problem. In particular, specific efforts must be committed to support studies that attempt to determine the molecular mechanism(s) of endogenous A β assemblies isolated by immuno-affinity combined with size-exclusion chromatography. In addition, there is an important need to standardize the relative concentrations of oligomeric A β used to assess their toxic properties. Only then, will we, as a field, reduce the amount of contradictory findings that plague our progression towards identifying specific signaling cascades and subsequent reasonable diagnostic and therapeutic interventions.

DISCUSSION

With growing evidence that at least 4 endogenous A β oligomeric assemblies, *i.e.* dimers, trimers, A β *56 and APFs, are produced in human and transgenic mouse brain tissue and are able to alter neuronal/cognitive function, it is now crucial that we establish the longitudinal profile of these molecules in human CSF and examine the relative relationship between the levels of these soluble A β assemblies and cognitive impairment. We must also focus our

combined efforts on identifying the respective origins of these four molecules. While A β trimers are not necessarily the most abundant species when amyloid burden is prominent *in vivo* (Lesne et al. 2006, Shankar et al. 2008, Shankar et al. 2009), they are preferentially produced and secreted by mature neurons *in vitro* (Lesne et al. 2006). In addition, trimers are detected at very early ages in APP transgenic mice (Lesne et al. 2006) and humans (Lesne et al. unpublished data) suggesting that A β trimers are the molecular brick for oligomers evading fibrillization. Supporting this hypothesis, larger A β oligomers detected in mice, such as potential hexamers (~27 kDa) and A β *56, are formed at ages following the formation of trimers and prior to plaque formation in Tg2576 (Lesne et al. 2006). Inspired by the terminology advanced by Dr. Glabe (Glabe 2008), we propose that trimers and trimer-based oligomers (hexamers and A β *56) should be referred as non-fibrillar A β oligomers. APFs would also be part of this subgroup following the recent demonstration that APFs do not give rise to fibrils (Lasagna-Reeves et al. 2011). In contrast, A β dimers start accumulating extraordinarily paralleling or slightly preceding the earliest deposition of amyloid at 8–10 months of age in Tg2576 ((Kawarabayashi et al. 2004); Figure 5B). Similarly in human brain tissue, dimers are only detected in subjects with elevated amyloid loads (Shankar et al. 2008, Lesne et al. unpublished data). It is therefore likely that dimers might either constitute the seed of amyloid plaques or might be generated within plaques. We therefore propose that A β dimers are oligomers capable of converting to protofibrils and fibrils. Further supporting this hypothesis, studies using the artificially generated A β S26C dimers revealed that toxic protofibrils form rapidly from A β dimers. Accordingly, we would advance that A β dimers should be termed as prefibrillar oligomers. This new classification of A β oligomers is illustrated in Figure 1 modeling the possible relationships between A β entities *in vivo*.

Another major source of potential discrepancy between studies relies on the use of protein mixtures containing multiple A β species. To minimize this effect, the specific mechanism(s) of action of purified A β assemblies should also be examined individually. Using SEC to separate various endogenous A β assemblies has become widely accepted and has been useful for the characterization and isolation of A β oligomeric species (Townsend et al. 2006, Walsh et al. 2005, Lesne et al. 2006, Cheng et al. 2007, Shankar et al. 2008, Reed et al. 2009). It would then be extremely powerful to demonstrate that studies performed with synthetic preparations can be replicated with endogenously produced A β oligomers of similar molecular weight as determined by SDS-PAGE and SEC for instance. Moreover, these same techniques will allow us to create new tools to detect A β oligomers with the hope that they will also being able to block A β from interacting with its potential receptors.

Fundamentally, the amyloid cascade proposed nearly 20 years ago and revised in 2002 (Hardy & Higgins 1992, Hardy & Selkoe 2002) has reached another level of complexity with the emergence of multiple deleterious A β oligomers. Based on the findings integrated here, it is possible that A β dimers, trimers, A β *56 and APFs may activate specific signaling pathways, with differential consequences on neuronal synaptotoxicity and survival. Complicating this view further is the possibility that the A β species might act at different times of AD pathogenesis. Drawing a parallel with Tg2576, trimers, A β *56 and dimers appeared to form in brain tissues in this sequence (the onset of formation for APFs is not known in Tg2576). Such experimental design might offer insights in better improving our understanding of oligomeric A β -induced toxicity.

To conclude, the hypothesis that A β oligomers constitute the initiator culprits of AD is holding up fairly well to our scientific scrutiny. In a larger context, the upcoming identification of novel antibodies specific to each A β oligomer or preventing the interaction of oligomeric A β with its receptors offers hope to individuals diagnosed with AD and to their families.

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Figure 1. Proposed model for endogenous oligomeric Aβ production

Based on our current knowledge, Aβ monomers and trimers are produced and secreted by neurons. Of note, the N-terminus of Aβ is color-coded in blue, the central domain of Aβ in white and the C-terminus of Aβ is shown in red. The assembling of Aβ molecules within oligomers was based on our observations of epitope availability using a large panel of Aβ antibodies (82E1, 6E10, BAM10, 4G8, 40- and 42-end specific Aβ antibodies, A11 and OC). Once in the interstitial fluid, Aβ oligomers follow a trimer-based expansion in size under physiological conditions/normal aging. These species evade fibrillar formation. Upon the presence of an unidentified factor, monomeric Aβ becomes misfolded allowing the peptide to enter the fibrillar oligomer pathway, whose first step corresponds to the formation of Aβ dimers. Dimers can rapidly expand to create dimer-based protofibrils, ultimately contributing to the formation of fibrillar Aβ as amyloid plaques.

Figure 2. Temporal accumulation of endogenous Aβ dimers in brain tissue of Tg2576 mice
(A) Western blots showing the relative levels of Aβ dimers detected following immunoprecipitation using formic acid extracts. Dimeric Aβ levels increase in parallel to plaque accumulation in Tg2576, suggesting that dimers are closely related to deposited amyloid. **(B)** Densitometry analysis of formic acid-soluble Aβ monomers and dimers in Tg2576. Empty circles represent monomeric Aβ while filled black circles correspond to Aβ dimers ($n = 3/\text{group}$). **(C)** Relative ratio between Aβ monomers and Aβ dimers in Tg2576. *, non-specific band due to the immunoprecipitation.

Figure 3. Comparison between endogenous A β species secreted by mature Tg2576 primary cortical neurons and 7PA2 cells

(A, B) Western blots showing the relative levels of A β oligomers detected with 6E10 following immunoprecipitation using 1 mL of conditioned medium. While dimeric A β levels is preferentially secreted by 7PA2 cells (B), A β trimers are produced and secreted by primary neurons derived from Tg2576 mice (A). The observed profile in neurons (A) suggests a trimer-based oligomerization process under physiological conditions.

Figure 4. Epitope mapping for Aβ*56 in Tg2576 soluble extracts

Immunoprecipitation/Western blots showing the detection profile of Aβ*56 present in Tg2576 mouse brains. A comparison between 6E10, A11 and OC antibodies (kind gifts from Rakez Kayed and Charles Glabe) is presented. The 6E10+/A11+/OC– profile characterizes Aβ*56 as a non-fibrillar Aβ oligomer. * indicate non-specific bands generated by the amplification process.

Figure 5. Relationship between endogenous low molecular weight Aβ oligomers and Aβ*56 in human brain tissue

(**A, B**) Linear regression analyses between the relative levels of soluble Aβ dimers (**A**) or trimers (**B**) and Aβ*56 detected in extracellular-enriched protein fractions of human brain tissue from the Religious Order Study ($n = 88$). Dimeric Aβ levels showed no association with Aβ*56 whereas the abundance of Aβ trimers was positively correlated with Aβ*56 brain levels. (Rho is indicated next to the P-value for Spearman Rank Correlation. Correlations were performed using the whole cohort, $n = 88$.)

Figure 6. Proposed model for the expression profile of endogenous Aβ oligomers in human brain tissue

We analyzed human brain tissue from 158 subjects with ages of 1 to 98 years at the time of death. Two cohorts were used for this study (Lesné et al., under review): 49 brain samples were from healthy subjects (from 1 to 62 years) and 89 from subjects with preclinical AD, MCI or AD. Briefly our findings indicate that in healthy subjects Aβ*56 was first detected in the 5th decade of life preceding elevations in Aβ trimers and dimers observed in the 6th and 7th decades. In preclinical AD, Aβ*56 appeared to reach a plateau. In MCI brains, Aβ*56 levels decreased significantly while Aβ trimers levels rose sharply. At this stage, brain levels of Aβ dimers slowly elevated. One possible explanation for this change in the relative expression of these species might be due to a destabilization of the non-fibrillar oligomerization pathway in favor of the fibrillar oligomerization pathway. Finally in AD brain tissue, Aβ dimers were increased while levels of Aβ*56 and Aβ trimers fell to discrete amounts. Such proposed scenario must need to be tested in longitudinal studies.

Figure 7. Correlation between extracellular levels of the oligomeric A β assembly, A β *56, and disease-relevant tau changes in AD brains

Using a very well cohort of the Religious Order Study, we measured A β *56 levels in the extracellular-enriched protein fraction as previously reported (Lesne et al., 2006) as well as soluble total and disease-relevant tau species (using Tau5 and Alz50 antibodies). All protein levels were normalized to the neuron-specific nuclear protein, NeuN, to compensate for neuronal cell death across groups. (A) Representative quantitative western blot for tau5/Alz50 in 30 subjects of our cohort (10 preclinical, 10 MCI and 10 AD) using a Li-Cor Odyssey system. (B) Quantification of soluble Alz50-tau levels revealed disease-relevant tau changes in MCI and AD brains. (C) Linear regression analysis between soluble Alz50-tau and A β *56 levels indicated a strong positive correlation in AD brains. Abbreviations: PC, preclinical AD; MCI, mild cognitive impairment; AD, Alzheimer's disease.

Figure 8. Receptors and signaling pathways potentially modulated by endogenous Aβ oligomers
 Among the various receptors proposed to act as oligomeric Aβ receptors, only a handful of postsynaptic receptors appear to be relevant to endogenously produced soluble Aβ entities: PrP^c, EphB2, RAGE, α7nAChR, Glutamate transporters, and possibly NMDAR. Since no endogenous Aβ oligomer has been directly shown to bind to NMDARs, and since most signaling pathways altered by naturally produced Aβ oligomers disturb physiological NMDAR transducers or NMDAR expression, Aβ oligomers might act as indirect modulators of NMDAR activity.