And now for something completely different: It's Amyloid-β with two subunits

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass diese Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Arbeit wurde bisher keiner Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht. Es wurde keine generative oder sonstige künstliche Intelligenz zur Hilfe genommen. Ich habe bisher keinen erfolglosen Promotionsversuch unternommen.

Düsseldorf, den 6. Juni 2025

Tis but a scratch.

- The Black Knight,
Monty Python and the Holy Grail

Danksagung

So eine Doktorarbeit ist nie kurz, aber bei mir hat es jetzt wirklich länger gebraucht, als zu Anfang erwartet wurde. Dann wiederum hätten wir alle Anfang 2019 mit unseren Vermutungen bezüglich der nächsten fünf Jahre ganz ordentlich daneben gelegen und ich habe sie, denke ich, gut genutzt.
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INHALTSVERZEICHNIS

Abstract

Amyloid oligomers are soluble multimeric species of misfolded protein that in the case of Amyloid-β (Aβ) and α-synuclein (αsyn) are not only neurotoxic but hypothesized to be the culprit behind the devastating neurodegenerative diseases Alzheimer's (Aβ) and Parkinson's Disease (αsyn). However, due to the innate transience of oligomeric structures, investigating their effect on cellular processes and their interactions with other disease-relevant protein structures is complicated. In this thesis, we apply the synthetic Aβ dimer dimAβ to 1. investigate the source of Aβ oligomers *in vivo*, 2. their effects on neurons and 3. their potential to inhibit αsyn secondary nucleation.

1. DimAβ as well as Aβ42 are shown to oligomerize faster at acidic pH and become more stable in oligomeric form as well as cluster in superstructures. These conditions can be found *in vivo* in the endo-lysosomal system in which both dimAβ and Aβ accumulate. This compartment has also been shown to harbor an elevated concentration of Aβ, which reaches the same order of magnitude as the decreased critical oligomer concentration of Aβ42 at this pH. In combination with the known leakage of Abeta aggregates from lysosomes and the release of smaller oligomers from pH-upshifted oligomer clusters, the endo-lysosomal system is inferred to be a major source of neurotoxic Aβ oligomers. 2. DimAβ is evaluated as an Aβ oligomer model by showing its effect on neurons, including tau missorting, a decrease of neuronal activity and dendritic spine binding. In this regard it shows the same effect known for Aβ, but is far more consistent and stronger than Aβ oligomers, likely due to its kinetic stabilization. 3. DimAβ oligomers are off-pathway oligomers that are known to not promote but inhibit Aβ aggregation. I show that they also inhibit αsyn aggregation in a concentration-dependent manner. This extends the model of off-pathway oligomer inhibition of amyloid aggregation to not rely on matching sequences but other shared properties, such as hydrophobicity.

Zusammenfassung

Amyloide Oligomere sind lösliche, multimere Spezies fehlgefalteten Proteins, die als Sackgasse und Zwischenschritt im Prozess der amyloiden Aggregation entstehen. Insbesondere im Falle von Amyloid-β (Aβ) und α-synuclein (αsyn) sind sie nicht nur neurotoxisch, sondern stehen auch im direkten Zusammenhang mit der Krankheitsentstehung von Alzheimer beziehungsweise Parkinson, zweier fortschreitender neurodegenerativer Erkrankungen, die gerade im Kontext einer alternden Gesellschaft zunehmend zum Problem werden. Trotz ihrer hohen Relevanz ist die Erforschung des Effekts oligomerer Spezies auf zelluläre Prozesse und ihrer Interaktionen mit anderen krankheitsrelevanten Proteinstrukturen nicht trivial: Oligomere sind inherent flüchtig, da sie im stetigen Gleichgewicht mit anderen Strukturen stehen. Dies führt unter anderem zur Entkupplung von Forschungsergebnissen, die *in vivo* beobachtet werden und solchen, die *in vitro* erzeugt wurden, da letztere mit deutlich erhöhten Konzentrationen arbeiten müssen, um ähnliche Effekte nachweisen zu können. In dieser Dissertation wird das synthetische, mit einem flexiblen Linker kovalent verknüpfte Aβ-Dimer dimAβ verwendet, um 1. die Entstehung von Aβ Oligomeren *in vivo* zu erforschen, 2. ihren Effekt auf Neurone zu konkretisieren und 3. ihr Potential zur Inhibition der sekundären Nukleation von αsynuclein zu zeigen.

1. In dieser Arbeit wird gezeigt, dass sowohl dimAβ als auch Aβ42 bei saurem pH deutlich schneller oligomerisieren und die bei neutralem pH später folgende Aggregation zu Fibrillen weitestgehend ausbleibt. Dies geht mit einer kinetischen Stabilisierung der Oligomere einher, die sich zudem in saurer Umgebung zu großen Superclustern zusammenlagern. Solche Bedingungen finden sich *in vivo* im endo-lysosomalen System, in dem Aβ akkumuliert und eine extrem erhöhte Konzentration aufweist, die sich in derselben Größenordnung befindet wie die minimal benötigte Konzentration, bei der Aβ42 *in vitro* Oligomere bildet. In Verbindung mit dem bekannten Entweichen von Aβ Aggregaten aus Lysosomen und der Feststellung, dass die Supercluster bei Neutralisierung der Umgebung wieder in die ursprünglichen Oligomere zerfasern, wird das endo-lysosomale System zu einer potentiellen Hauptquelle von Aβ Oligomeren.

2. DimAβ wird als Modell für Aβ Oligomere evaluiert, indem es verwendet wird, um den Effekt von Aβ Oligomeren auf Neurone zu verdeutlichen: Es wird das Phänomen des “Tau missortings“ beobachtet, das die veränderte Verteilung des Proteins Tau innerhalb von Neuronen beschreibt und unter anderem im Kontext von Alzheimer beschrieben wurde; ein Abfall der neuronalen Aktivität und das Binden an dendritische Dornen wird ebenfalls gezeigt. Damit spiegelt dimAβ das Verhalten von Aβ, mit dem Unterschied, dass die Resultate an dieser Stelle konsistenter und teilweise stärker ausgeprägt waren. Dies wird mit der kinetischen Stabilisierung von dimAβ begründet und dem deutlich höheren Anteil an oligomeren Spezies.

3. DimAβ bildet “off-pathway“ Oligomere, also solche, die nur mit Monomer im Gleichgewicht stehen und sich nicht direkt zu Fibrillen weiterentwickeln können. Es wurde bereits zuvor festgestellt, dass sie die Aggregation von Aβ und insbesondere dessen sekundäre Nukleation inhibieren. In dieser Arbeit wird aufbauend darauf gezeigt, dass dieses Phänomen nicht auf Fibrillen derselben Spezies beschränkt ist, sondern dass dimAβ auch die sekundäre Nukleation von αsynuclein inhibiert. Dies lässt den Schluss zu, dass nicht Sequenzähnlichkeit, sondern andere Faktoren wie zum Beispiel Größe und Hydrophobizität von off-pathway Oligomeren für diese Interaktion verantwortlich sind.

1

INTRODUCTION

At the time of writing this thesis, a science scandal has erupted around fraudulent data produced and published by Eliezer Masliah spanning multiple decades (Elliott, 2025). He falls in line with other misconduct cases such as Berislav Zlokovic and Domenico Praticò and highlights, in the opinion of the author, not only his own moral weakness, but also the immense public interest in the topic and subsequent pressure on scientists to produce results. Neurodegenerative diseases are a sword of Damocles hanging over an ever-aging population, so the need for a remedy far outstrips the actual progress being made. The research of neurodegenerative diseases is complicated - it involves the brain, which if easily understandable would render us incapable of understanding it. It is important not to lose sight of the bigger picture when delving into small details, neither the scientific subtleness nor the human fates (Schützmann, 1995). The introduction to this thesis is therefore going to try and convey not only the specific topics needed to understand the research that has been done but also the broader surroundings to be able to contextualize them.

Amyloid Diseases

The category of human diseases called amyloid diseases is named after a microscopic feature either triggering or at least accompanying them; that being highly ordered, β-sheet-rich protein structures called amyloid fibrils. A name that is sometimes used interchangeably with amyloid disease is the term “amyloidosis“. However, especially within medical literature, this mostly refers to diseases that harm the whole system (e.g., Multiple System Atrophy) or certain tissues or organs (e.g., atrial amyloidosis) by an overload of fibrils, rendering this expression not distinct enough. In this thesis it will therefore not be used in a general sense.
Roughly half of all amyloid diseases are neurodegenerative diseases, most notably Alzheimer’s (AD) and Parkinson’s disease (PD) (Chiti and Dobson, 2017). This high portion of neurodegenerative diseases will gradually decrease over time since with more awareness towards the incidence of amyloid fibrils in diseases other than those of the brain, some that were not initially categorized as amyloidogenic in nature, have been added to the list of amyloid diseases such as Type 2 Diabetes (T2D) (Westermark et al., 1992).

Alzheimer’s Disease

Alzheimer’s disease is the most common form of dementia in Western societies (Hardy and Selkoe, 2002). It is a progressive, chronic, and unremitting neurodegenerative disorder whose incidence rate generally scales with age.

About 5% of AD cases represent early-onset familial forms (EOFAD) that are due to mutations in the genes for the presenilins (*PS1* and *PS2*), the amyloid precursor protein (APP) (Clarimón et al., 2009; Lleó et al., 2002), *SORL1* (Pottier et al., 2012) and at least one of the genes for APOE (*APOE4*) (Fortea et al., 2024). The majority of cases are sporadic, and an unambiguous cause has not yet emerged, even though genetic factors play a huge role (between 60-80%) (Gatz et al., 2006).
However, connections have been made to a multitude of different risk factors: A set of infectious agents from viruses such as the Epstein-Barr-virus over bacteria like Chlamydia to single-cell eucarya such as *toxoplasma gondii* have been observed to increase the risk for AD (Piekut et al., 2022). A potential role of environmental factors has also been hypothesized, such as physical activity, where a higher level of exercise correlated with less cognitive decline and a smaller amyloid burden (Valenzuela et al., 2020). Lipid metabolism dysregulation is also implicated in being a strong risk factor for AD, since the lipidation status of APOE is directly connected to its ability to bind and clear Aβ (Hanson et al., 2013). Obesity is an AD risk factor (Al-Kuraishy et al., 2023; Golan Shekhtman et al., 2024). A higher education correlates with a greater tolerance for amyloid plaques on the brain function (Bennett et al., 2003). Exposure to pesticides is implicated with a higher risk for AD (Yegambaram et al., 2015), as is smoking (Zhong et al., 2015).
Genetically, being a carrier of at least one APOE ε4 allele is associated with a 3-4 times higher AD risk (van der Lee et al., 2018), but there are about 40 different other variants that contribute to AD risk (Jansen et al., 2019). A lot of these risk genes are implicated in the microglia response, however, it is not clear whether this is aimed at Aβ-plaques only or also interacts with tau pathology (Leyns et al., 2017; Sierksma et al., 2020).
Based singularly on external symptoms, AD is hard to differentiate from other dementias (Knopman et al., 2001) and even though the gold standard for diagnosis is still *post-mortem* examination of the brain, less invasive and more useful methods have emerged, such as measurement of Aβ- and tau-levels ("Alzheimer's disease CSF profile") in cerebrospinal fluid (CSF) (Maddalena et al., 2003) and Aβ PET imaging (Ossenkoppele et al., 2015). Both a positive Aβ PET scan and a low Aβ42 level can support an AD diagnosis, and both are detectable more than 15 years before the onset of dementia (Fagan et al., 2007; Toledo et al., 2013).
Another useful approach is the combination of different biomarkers consisting of Aβ (A), phosphorylated tau (T), and neurodegeneration (N), which was recently amended by inflammatory/immune mechanisms (I), vascular brain injury (V), and α-synucleinopathy (S) (Jack et al., 2018, 2024). These methods are based on the main characteristic of AD, neurodegeneration, and its two main potential triggers: The peptide amyloid β (Aβ), which accumulates into plaques and other, smaller but likely more neurotoxic species (see p. 17), and the protein tau, which gets hyperphosphorylated and forms intracellular neurofibrillar tangles that correlate well with neuronal decline (see p. 21). The spread of Aβ pathology can be classed in Braak stages, running from A to C (see fig. 1.1). As a quick note to prevent confusion, it should be mentioned that both diseases, AD and PD, make use of staging schemes called Braak stages, which are functionally distinct in each disease. While Braak first introduced them for AD, they have gone on to be more prominent for the use in PD.
Another hallmark of AD is neuroinflammation, which is defined as an immune system stimulation in the central nervous system (CNS). It is mediated by microglia activation that initially counteracts both Aβ accumulation and tau hyperphosphorylation (Gratuze et al., 2018; Merlo et al., 2018), but it can, in turn, cause neurodegeneration in later stages (Fan et al., 2017). This contradictory behavior can be explained by the spectrum of microglial activation from anti-inflammatory phenotype M2 to pro-inflammatory phenotype M1 (Pawelec et al., 2020). For

the longest time, neuroinflammation has been treated as a consequence of neuronal injury by either Aβ or tau accumulation (Heneka et al., 2015; Kempuraj et al., 2016). However, it may not only precede many pathological changes (Hamelin et al., 2016) but is also involved in a vicious circle with both Aβ- and tau-pathologies (Hanslik and Ulland, 2020; Wang et al., 2016).

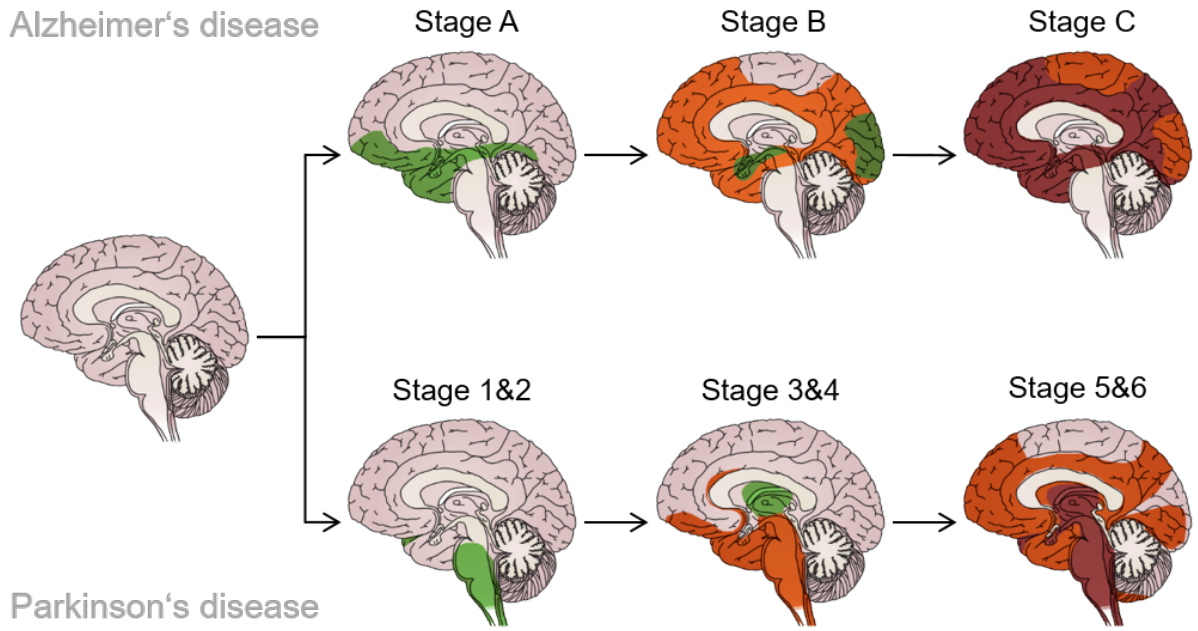

Figure 1.1: The Braak staging scheme for the spread of pathology in AD and PD. For AD, it is based on tau NFTs and Aβ plaques; for PD, it is based on αsyn Lewy body pathology. The colors represent the severity of the pathology from green to orange to red. Adapted from Braak et al. (2003) and Masters et al. (2015).

Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder that affected roughly 12.3 million individuals worldwide in 2024, with a steep increase from 6.1 million in 2016 (Zhu et al., 2024), second only to AD. Clinically, patients with PD present with a mix of motor symptoms such as bradykinesia with either rest tremor, rigidity, or both, and non-motor symptoms such as olfactory loss, cognitive decline, depression, pain, REM sleep behavior disorder, cardiac sympathetic denervation, and constipation (Postuma et al., 2015). There are no definitive tests that can be used to diagnose PD, only to help exclude confounding disorders (Bloem et al., 2021). PD, on average, progresses slower than AD, lengthening the time people have to either live with PD or care for patients with PD (Macchi et al., 2020). Multiple staging systems therefore exist to categorize the extent to which a person suffers from PD: The Hoehn-and-Yahr-scale that relies on motor symptoms and degree of disability (Hoehn and Yahr, 1967); the PANDA instrument that screens for mental decline and depression (Kalbe et al., 2008); and the Mini-mental state, which can be used to grade the cognitive state of psychiatric patients as well as those with PD (Folstein et al., 1975).

Judging from the clinical picture alone, several disorders can mask as PD. They are often referred to as "(atypical) parkinsonism" or "parkinsonian disorder" but are distinct from PD. PD is defined biologically by the presence of Lewy bodies (LB), proteinaceous accumulations in neurons that mainly consist of a protein called α-synuclein (αsyn) (see p. 14). This Lewy pathology was

first and is most prominently observed in the brain, following a spreading pattern also called Braak stages (see fig. 1.1)). It leads to degeneration of the dopaminergic neurons, even though the exact process is not yet completely clear.
More recently, αsyn accumulation has also been observed in other parts of the body, most notably the gut, leading to an increasing definition of PD as a multisystemic and multiorgan pathology (Costa et al., 2023). While the starting point of PD was hypothesized at first to be in the CNS, a newer hypothesis advocates for the olfactory bulb and vagus nerve to be involved in the spread of αsyn pathology from the gut to the brain (Hawkes et al., 2007), which is supported by a heightened risk for PD in patients with inflammatory bowel disease (Brudek, 2019). Sometimes, these are referred to as brain-first and body-first models (Just et al., 2022).
Among many hypothesized risk factors for PD, the most convincing alongside old age are exposure to pesticides, which might explain the rapid increase in PD after World War II (Ratner et al., 2014; Tanner et al., 2011) and head injury (Camacho-Soto et al., 2017). Apart from sporadic PD cases, there are a small number of genes that are univocally linked to PD and its early-onset forms (before age 50 or even 40), especially *SNCA, LRRK2, PRKN, PINK1,* and *GBA*, which are each linked to a distinct phenotype (Bloem et al., 2021). *SNCA* is the gene coding for the protein αsyn, which is assumed to be the main culprit in the pathogenesis of PD (see p. 12).

Motivation

According to recent data, AD prevalence is going to double from 2019 to 2050, aggravating the burden on especially Western societies (GBD 2016, 2018). The therapy of patients with AD is focused on the management of the symptoms rather than treatment due to the well-nigh absence of effective drugs that target the underlying problem (see p. 23 for new but largely unproven treatment options). It encompasses cognition-enhancing agents that combat neurotransmitter abnormalities (cholinesterase inhibitors and NMDA receptor antagonists (Masters et al., 2015)) and psychotropic drugs used for behavioral changes, most of which are used off-label and exhibit considerable adverse effects (Schneider et al., 2006).
PD prevalence has risen rapidly in the last years, so much in fact that some refer to this growth as the “Parkinson pandemic“, due to it not only being age-related and therefore assuming other causes (Dorsey et al., 2018). While PD is treatable, there is no cure for it, with the drugs being prescribed only alleviating the symptoms but not influencing the root cause. Therapeutics are comprised of dopamine analogues, precursors (of which the most prominent and agreeable is Levodopa), or chemicals interacting with dopamine, its receptors, or its degraders, and of drugs for the treatment of distinct motor and non-motor symptoms. Other invasive interventions for motor-symptoms (deep brain stimulation or ablative neurosurgery) also exist (Foltynie et al., 2024).
Both the looming increase in cases of neurodegeneration in an ever-aging population and the as of yet virtually non-existing options for curing both diseases underscore the importance of researching the molecular mechanism underlying both AD and PD: amyloid aggregation, the formation of prion-like, misfolded proteins that interfere with normal brain chemistry.

Amyloid Aggregation

Amyloid aggregation describes the process of assembling proteins into highly organized and dense non-covalent polymers termed amyloid fibrils that normally do not correspond to the active or physiological state of the protein used as building blocks (Willbold et al., 2021). Strict

geometric requirements (Trovato et al., 2006) distinguish these aggregates from the kind of amorphous, insoluble misfolded structures that are normally sought to be avoided for instance in heterologous protein production (Goode et al., 2021). In the context of this thesis, the term *aggregate* therefore refers exclusively to amyloids.
The term originates from the fibrils' capacity to be stained by iodine in a similar manner to starch, which led Rudolph Virchow in 1854 to assume having found starch in peculiar cerebral tissue (Sipe and Cohen, 2000) and calling it "amyloid" (from Greek *amylos*, starch). Nowadays, the dye most often used to monitor the formation of amyloids *in vitro* is Thioflavin T (ThT), a fluorescent benzothiazole with an affinity for β-sheet-rich structures (see p. 6). A typical *de novo aggregation assay* is therefore a setup that combines the protein in the desired conditions with ThT to monitor the occurrence of fibrils or other β-sheet-rich structures. A curve obtained by plotting the fluorescence intensity over time in such a manner normally features a lag time after which the signal rises in a near-sigmoidal manner until it reaches a plateau. This assay can be modified by *seeding* the protein with preformed fibrils, thereby eliminating the lag time in favor of a half parabola with a vertical directrix. Due to some aggregation assays taking multiple days, sometimes a poisonous substance such as NaN_3 is added to prevent bacterial growth (Wördehoff and Hoyer, 2018).
While it has been theorized that possibly all proteins have the potential to form amyloids (Iadanza et al., 2018), only relatively few have hitherto been found to be pathologically relevant (42 in 2022 (Buxbaum et al., 2022)). The diseases associated with amyloid aggregation are called amyloidoses and can be roughly divided into neurodegenerative (affecting the central nervous system, such as Alzheimer's Disease) and systemic (affecting the rest of the body, such as light chain amyloidosis). However, when referring to systemic amyloidoses, the prefix is often omitted. They are generally treatable but not curable, and most of them can be fatal.

The Amyloid Fibril

The endpoint of amyloid aggregation is called an amyloid fibril or **fibril** for short (see fig. 1.2). It is an elongated structure built from single layers consisting of usually two protofilaments (for most pathological fibrils, but there are functional amyloids with three filaments per layer (Hervas et al., 2020)). Each layer either strictly or almost adheres to a $C_{2(*)}$-symmetry; in latter cases, the protofilaments are slightly out of level along the fibril axis in relation to each other. In either case, a protofilament usually consists of multiple β-sheets linked by loops and turns, which form β-strands that run orthogonal to the fibril axis, and most times, span the middle of the protein sequence, leaving the ends soluble and quasi-coating the fibrils. They are stabilized by a variety of interactions, such as dry steric zippers between side-chains (Eisenberg and Jucker, 2012), which need not feature self-complimentary sequences but can also be heterosteric zippers; polar zippers made from interdigitation of polar side chains (Falcon et al., 2018); solvent channels (Falcon et al., 2019); β-turns interconnected by backbone-sidechain or sidechain-sidechain interaction and hydrogen bonds (Radamaker et al., 2019). Since the β-sheets stack in parallel beyond the layers, the interactions with the upper and lower layer also stabilize the individual layers as well as form a superstructure termed cross-β-structure, which runs along the fibril axis. Some fibrils are flat while others are twisted, referring to the displacement of the individual layers in relation to one another, but even the twisted fibrils have a rather slow twist in comparison to, for instance, DNA.
The exact arrangement on protofilament level is protein- and fibril strain-dependent, even though some motifs exist. **Fibril strain** refers to the polymorphisms found in fibrils of one and the same primary structure: Either due to solution conditions, posttranslational modifications, unknown

factors, or just because of energetically nearly equivalent arrangements, the exact limits and connections of the β-sheets can vary (Bousset et al., 2013; Ma et al., 2016; Sidhu et al., 2014). In one and the same fibril, however, each layer always follows the same arrangement. In this regard, a fibril could be seen as a protein crystal that can only grow in one dimension. For some proteins, a correlation between the form of disease and fibril structure has been established; for others, no such relationship has been discovered.

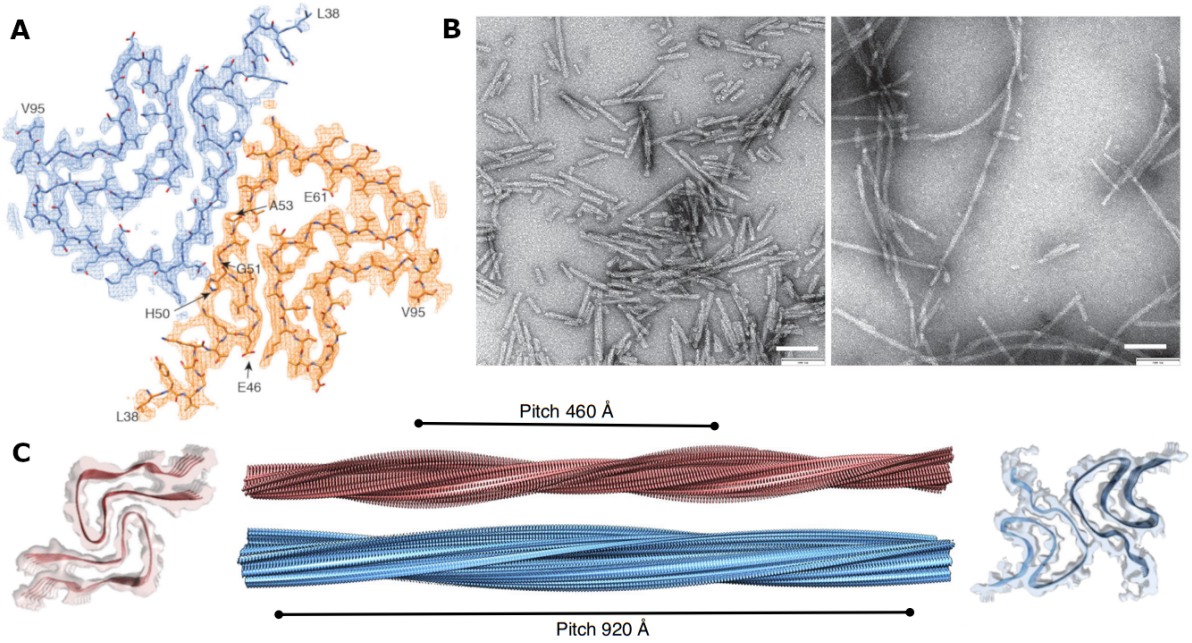

Figure 1.2: Examples of the structure of amyloid fibrils. In (A), the fibril core of an αsyn fibril is shown as an electron density with inlaid protein structure. Negative stain TEM images (B) and distinct fibril arrangements (C) of αsyn strains show variety in the same amino acid sequence. Adapted from Guerrero-Ferreira et al. (2020) and Li et al. (2018).

Mainly for this reason, a divide in the amyloid field exists between data that have been compiled *in vitro* and data from *in vivo* sources, since they exhibit differing polymorphs (Annamalai et al., 2016; Bansal et al., 2021). For disease-specific structures, it seems essential to work as close to the physiological system as possible, prioritizing *in vivo* conditions to the artificial *in vitro* ones, especially if the aim is to find interaction and inhibition partners for them for therapeutic purposes (Melki, 2015). For the elucidation of the aggregation process, however, falling back onto the better controlled and understood *in vitro* assays was and is necessary. The direction of the field is definitely leaning more towards *in vivo*-generated data.
Amyloid fibrils are partially protease-resistant (Frare et al., 2006; Nordstedt et al., 1994) due to the dense packing of their core. They have remarkable physical properties such as a hardness in the lower range of steel and a mechanical stiffness half of what is known for silk (Smith et al., 2006) and are therefore interesting subjects for material science.

Amyloid dyes: Chemicals that interact specifically with amyloid structures are important for both tissue-staining of biological samples and for enabling the monitoring of *in vitro* assays. Although technically, the first dye used for amyloids is iodine, its potential for use is both too limited and not sensitive enough.
In 1922, Congo Red (CR, also known under direct red 28) was used for the first time for the express purpose of staining amyloids even though it had been used in the context of microscopy

before (Howie and Brewer, 2009). CR is a symmetric bisazo dye with a conjugated π-electron system consisting of two naphthalene and two benzene rings (see fig. 1.3 B). It can act as a pH indicator, changing its color from red in an alkaline environment to blue under acidic conditions starting from lower than pH 5 (Mera and Davies, 1984) due to the protonation of either the primary amines or the azo groups removing free electron pairs from the π-system (Bonancêa et al., 2006). This system is also the reason for CR self-organizing into rod-like micelles in aqueous solution via π-stacking (Woodcock et al., 1995) as well as attaching to surfaces such as cellulose and, more importantly, amyloid fibrils.
CR not only stains amyloid fibrils, it hereinafter exhibits a phenomenon called (linear) birefringence. This term describes the retardation of polarized light by an anisotropic material (a material with a spectrum of refraction indices), resulting in elliptically polarized light that can be detected by rotating another polarizing filter in front of the detector. CR orients itself on the surface of amyloids in a way that induces this anisotropy and therefore birefringence. Interestingly, in most publications making use of this property, it is used in conjunction with a color such as “apple-green birefringence“, even though this color is neither explainable by this distinct phenomenon, nor is it the only one that regularly appears (Howie and Brewer, 2009).

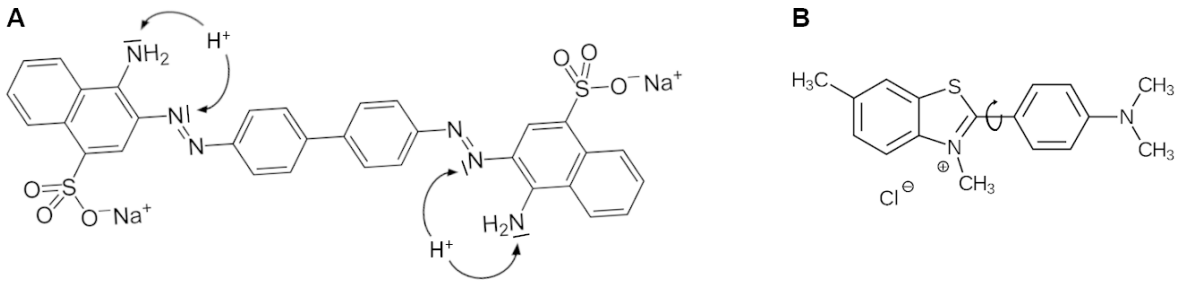

Figure 1.3: The structures of the most prominent amyloid dyes, Congo Red and Thioflavin T. In A) Congo Red is depicted in its deprotonated state with the likely targets for protonation. In B) Thioflavin T is shown with the axis for rotation highlighted.

Since the usage of two polarizing light filters is rather cumbersome, the usage of CR has declined in favor of Thioflavin T (ThT), a benzothiazole which exhibits fluorescent properties when bound to amyloid fibrils. In solution, the molecule has a rotational axis in the single bond between the two rings, separating the two π-systems (see fig. 1.3 B), but upon binding to a β-sheet-rich flat surface, the steric constraints connect both systems, resulting in a marked increase in quantum yield with an excitation maximum of 450 nm and an emission maximum of 482 nm (Krebs et al., 2005). This then highlights both amyloid fibrils and also certain β-sheet rich oligomeric structures.

The Amyloid Aggregation Process

The first step of amyloid aggregation, called **primary nucleation**, is defined as the formation of an initial small oligomer in the absence of already existing fibrils (see fig. 1.4). It is the most elusive part of the process due to the high(est) Gibbs free energy and accordingly low population of these initial oligomers (Kashchiev and Auer, 2010). An initial oligomer that is stable enough to be more likely to go on along the aggregation pathway than to dissolve is called a nucleus. This likelihood is highly dependent on the (local) concentration of monomers: Below a critical concentration, the energy needed to transfer a monomer from soluble into an aggregated state is

positive, and the process is therefore unlikely; above said concentration, it becomes negative. This step can occur both homogeneous and heterogeneous. *Homogeneous primary nucleation* occurs in solution, and therefore, in addition to energetic concerns, the larger the minimal number of monomers needed for the formation of these nuclei, the lower the probability of these meeting in solution becomes; this probability also benefits from a higher (local) concentration (Sarić et al., 2014). It is contrasted with *heterogeneous primary nucleation*, referring to the critical nuclei not forming in solution but on a surface that is not made up of the same monomers as building blocks. These could be different proteins, but also interfaces such as air-water, lipid vesicles or -membranes, or simply the inner wall of a multiwell-plate used in an assay (Campioni et al., 2014). The energetic advantage of heterogeneous primary nucleation is due to the second participant often being either much larger than a monomer or (nearly) stationary and having a certain affinity for the monomers. It could therefore be regarded as a form of surface catalysis. This leads to a rise in local monomer concentration and will therefore increase the probability of nucleus formation. Due to this, even seeded aggregation with non-competent seeds normally cuts lag time down.

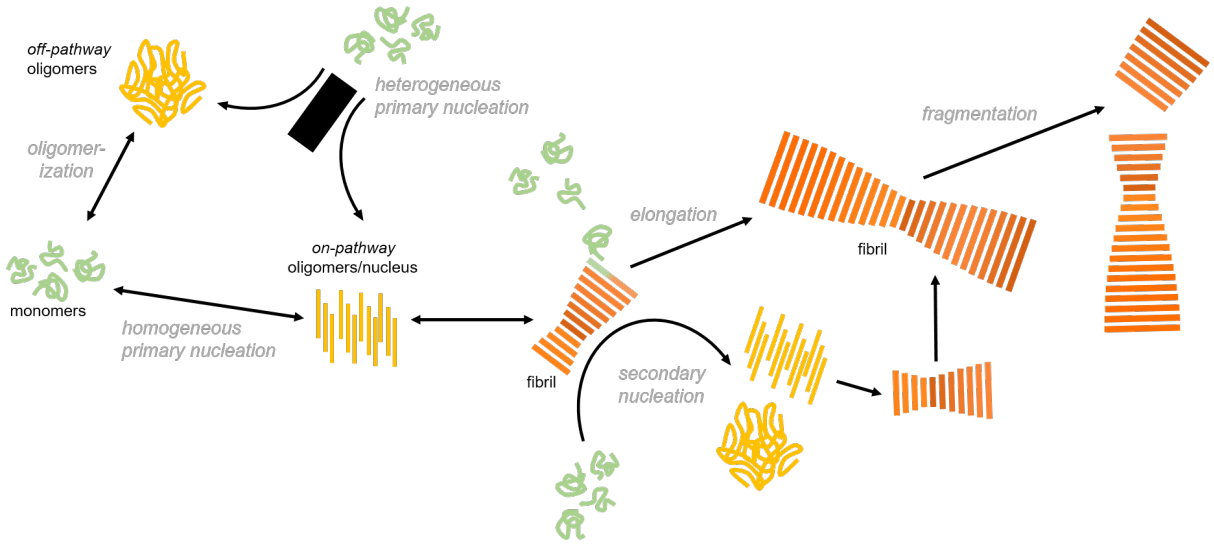

Figure 1.4: The many processes comprising amyloid aggregation. Monomeric species are shown in green, oligomers in yellow and fibrils in orange.

If the surface needed for heterogeneous primary nucleation is a fibril made up of the same monomer as building blocks, the process is usually called **secondary nucleation**. This process is quite common outside of the amyloid field; for instance, the crystallization of a chemical compound by the addition of a previously formed seed induces secondary nucleation, which leads to the generation of an exactly replicated structure (Palermo, 1968). In contrast to this, however, secondary nucleation in fibrils does not generally preserve the strain (Hadi Alijanvand et al., 2021). In fact it can even generate completely different structures, such as oligomers, which makes this process not only dangerous due to the exponential propagation of fibrils but also due to oligomeric species generally being regarded as more toxic (Cohen et al., 2013; Törnquist et al., 2018; Xu et al., 2024).

Once nuclei have been generated, they will grow linearly by adding monomers, either forming off-pathway oligomers or likely undergoing a conformational shift and forming fibrils. The process in which a fibril recruits monomers to its ends and grows is called **fibril elongation**. This encompasses the diffusion of a monomer to the fibril end, the change of its conformation and the incorporation into the fibril. The available data allude to this process not having an

intermediate step that changes the conformation of the monomer before engaging the fibril, but rather disordered monomers docking and changing structure afterwards at a slower pace (Bacci et al., 2017). It has been shown that only a fraction of fibril ends elongate at the same time, likely because some fibril ends become non-competent or inactive by the incorrect folding of an added monomer unit (Pinotsi et al., 2014). The speed of elongation and nucleation processes is highly dependent on solution conditions such as pH and ion strength (Buell et al., 2014). A fibril can either persist, slowly disassemble starting at its ends, or break. This last process is called **fragmentation** and doubles the available ends and more than doubles the active ones, which is especially dominant in *in vitro* shaking assays.

Amyloid Oligomers

During the aggregation process, a multitude of additional structures, comprehensively called oligomers, can form either as intermediates or as byproducts. Oligomers are most easily defined by their complement. They are neither monomers in whichever state, whether unfolded or folded, nor are they highly-ordered β-sheet-rich fibrils or insoluble protein aggregates. Every structure in between these extremes could be called an oligomer (and will be in some publication). The size has an upper limit though indicated by the prefix “oligo“ (greek for few, normally up to 1000 units) being used instead of, for instance, “poly“; and a lower limit since functional di-, tri- or tetramers are usually also not included in the context of amyloids. This ambiguity hampers the contextualization of published data even when focusing on a small number of specific proteins. In the following, most information will therefore be separated by specific protein (see p. 15 for αsyn and p. 20 for Aβ).
In general, oligomers are either on- or off-pathway in regard to their ability to directly, without degrading back into monomers, mature into fibrils (on-pathway) or lack thereof (off-pathway). There are three different timepoints in aggregation that might yield oligomeric species:

1. They might be primary oligomers, which are directly being formed from monomers, before either evolving into fibrils (on-pathway) or disassembling back into monomers (off-pathway).
2. They can form as a byproduct during secondary aggregation processes, as either intermediates to fibrils (on-pathway) or as non-seeding misfolded species (off-pathway).
3. They can originate from the fragmentation from afore formed fibrils (Xue et al., 2009), which would make them on-pathway by definition. This possibility is mostly discussed as a possibility for the seeding capacity of fibrillar aggregates (Lam et al., 2016). It is also to be expected that they have structurally more in common with fibrils than other oligomers.

On-pathway oligomers are in equilibrium with monomers and with fibrils, depending on their propensity to cross the energy threshold for aggregation. In this context, sometimes the term “critical nucleus“ is used to describe an on-pathway oligomer that is as likely to go on to become a fibril as it is to disintegrate, in accordance with classical nucleation theory (Sear, 2007). The on-pathway kind of oligomer is distinctly short-lived and hard to detect and characterize (Lee et al., 2011).

Off-pathway oligomers are only in equilibrium with monomers and so canonically inhibit fibril growth by depleting the monomer pool. In addition to this, they can bind to fibril surfaces and inhibit secondary nucleation events (Hasecke et al., 2021). It should also be noted that the amount of fibrillar aggregates does not necessarily have to correlate with the number of off-pathway oligomers present, which is especially relevant since plaque load in neurodegenerative diseases

is not always a good predictor for neurological decline. If they are primary oligomers, they can form independently from fibrils; *in vitro* it has already been shown that primary oligomer formation is dependent on a "critical oligomer concentration" of monomers (Hasecke et al., 2018).

Independent from their exact origin, amyloid oligomers are metastable species that are hard to isolate, especially because they are in equilibrium with monomeric species, any method that traps them risks changing their structure and size (Shea and Daggett, 2022). The size seems to correlate to their ability to interact, permeate, and cross the plasma membrane, with earlier, smaller species being more potent in this regard (Campioni et al., 2010). In *in vivo* studies, oligomers are often located by multimer-specific antibodies that cannot differentiate between different kinds of assemblies, so the exact kind of oligomer is often unclear.

Aggregation in the Context of Protein Folding

In essence, since fibrils are formed by folded amino acid chains, amyloid aggregation is a type of protein misfolding. And since the amyloid field has originated in the field of protein folding, it initially borrowed a lot of its concepts and nomenclature from this area of research. However, the field has evolved past that and has expanded its previous assumptions.
The field of protein folding (Dill and MacCallum, 2012) is concerned with the mechanisms and endpoint of the initially 1D protein sequence of amino acids intertwining until it reaches a functional 3D structure. This process is largely self-consistent and extremely fast; all the more incredible that a protein with a specific primary sequence folds itself into a distinct shape. The most obvious assumption that amyloid aggregation violates is therefore the dogma that all the information needed for a functional three-dimensional structure is encoded in the primary sequence of a protein (Anfinsen, 1973) (the existence of a one-to-one sequence-to-structure relation). Since most proteins are theorized to form fibrils under the correct conditions (Iadanza et al., 2018) but also have a (different) functional structure, this correlation is not as strong as previously thought. Distinct fibril strains make this even more evident.
A conundrum of protein folding is the Levinthal's paradox, which states that the immense number of possible theoretical conformations of a peptide should make it impossible to reach its desired structure in any realistic timeframe (Levinthal, 1968). However, proteins sometimes fold in only microseconds. This difference is normally explained by placing the different folding states of a protein on an energy landscape that has a funnel-like shape, with the functional structure having the least energy (see fig. 1.5). This encompasses a local-to-global approach to folding in which smaller, adjacent parts of the protein lessen their energy by folding into structural motifs such as α-helices and β-sheets, which begin interacting until they reach the minimum of the funnel. Amyloid fibrils expand this model by adding a second local and arguably global energy minimum corresponding to the fibril state. Depending on the protein, the two funnels are either separated (if the monomer needs to unfold completely before aggregation is possible) or partially merged (if the monomer has less of a kinetic barrier to refold).
In contrast to spherical and other folded proteins, which display secondary and tertiary structures that are stable over time in their functional form, some proteins lack both. Intrinsically disordered proteins (Mukhopadhyay, 2020) (IDPs) can indeed form secondary motifs but do so on such small time scales that they appear as random coils if observed by most methods. In some cases, they adopt a structure upon binding to another molecule and are functional as such. This makes IDPs, in general, more flexible and versatile but also poses the risk of folding into fibrils: Their native state is not confined by energy barriers preventing them from "falling" into the fibril

funnel. In fact, some of the peptides linked to debilitating neurodegenerative diseases are IDPs, such as α-synuclein and Amyloid-β.

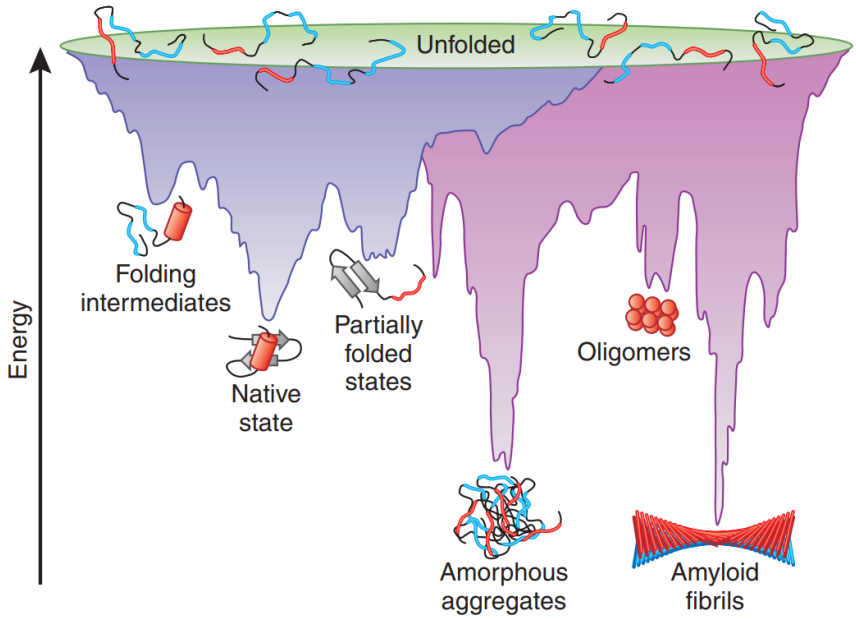

Figure 1.5: Schematic depiction of the energy landscape of protein folding. Adapted from Hartl and Hayer-Hartl (2009).

Aggregation in the Context of Prions

For a long time, prions have been misfolded structures of the PrP^C (cellular prion protein native to the host) that adopt an alternative, oftentimes substantially different conformation from their functional state (PrP^{Sc}), which then auto-catalytically self-replicates by converting native-state protein units into the prion fold. These prions are pathologically relevant for a number of diseases like Kuru or Creutzfeld-Jacob, which are not only fatal and not treatable but also transmissible. Amyloids share most properties of prions. The most obvious one is the self-propagation through the consumption of same-species monomer units. Both have a penchant to form β-sheet-rich structures, which then behave in an aberrant way. Both prions and amyloids have transmissive potential, and both exhibit strains which can have an influence on disease progression. Therefore, it has been proposed therefore to view amyloids as prions and to standardize nomenclature (e.g., to refer to aberrant αsyn structures as αsyn prions) (Asher et al., 2020; Walker and Jucker, 2015).

There are, however, some differences in these shared properties, which might warrant the classification of amyloids as a subset of prions rather than equating both species. Amyloid species are a lot less infectious. For instance, no cases are known of accidental transmission of amyloid diseases from one organism to another, while there are some prion diseases that spread inexorable (a good example is chronic wasting disease in deer). In laboratories, one can transmit amyloid disease pathology with considerable effort (Kordower et al., 2008; Mendez et al., 2008), but in general, the transmissibility of amyloid pathology is limited to intercellular spread in the same organism (Hansen et al., 2011). Additionally, there are a lot of amyloidogenic proteins where a direct correlation between fibril strain and pathology has yet to be observed.

Another aspect which will play a role in the adoption of new nomenclature yet to come is the

potential for confusion: What has classically been called prions are misfolded species of exactly one protein and its isoforms, which has been named accordingly *the* prion protein. In contrast to this, amyloid refers to many different proteins with the shared potential to form a certain fold.

α-synuclein

α-synuclein (αsyn) is mostly expressed in the human brain, especially the *substantia nigra, hippocampus, neocortex, thalamus,* and *cerebellum* (Yu et al., 2007), but can also be found in astonishingly high concentrations in platelets and erythrocytes (Barbour et al., 2008). It is implicated in the pathogenesis of PD, not only because of its presence in Lewy bodies (see p. 14) but also because of the existence of several familiar mutations that lead to early-onset PD (see p. 12). Its aggregated form has also been found in roughly half of AD patients (Arai et al., 2001; Hardy and Higgins, 1992). In the brain, αsyn makes up to 0.5-1% of total cytosolic protein (Iwai et al., 1995), so according to the inverse relationship of aggregation rate and expression level (Tartaglia et al., 2007), it should not be very aggregation-prone. While this hypothesis holds true in general, the particular segmentation of αsyn leads to a different behavior in reality.

Structure

αsyn is 140 amino acids long and can be subdivided into three distinct domains: The N- and C-terminus and the NAC-region. In isolation, it presents as an intrinsically disordered protein with only a loose association between N- and C-terminus (Stephens et al., 2020).

N-Terminus: The N-terminus (1-60) is overall positively charged and features four repeats of the motif "KTKEGV", a fifth one reaching into the second domain (see fig 1.6). Up to four more degenerated repeats can be localized (Brontesi et al., 2023) further down along its sequence.

MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAE
KTKEQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQLGKNEEGAPQEGILE
DMPVDPDNEAYEMPSEEGYQDYEPEA*

Figure 1.6: The sequence of αsyn. The highly conserved KTKEGV repeats are marked in green, the worse-conserved in yellow, and the degenerated in red.

The N-terminus is mostly mentioned in interaction with other molecules: It does so by the aforementioned repeats forming alpha-helices that can then bind to (mostly negatively charged (Stöckl et al., 2008) or unsaturated (Broersen et al., 2006)) lipids, of which the brain offers plenty (Chen et al., 2008). Since the helices have a higher affinity for curved membranes, they prefer binding to vesicles (Pranke et al., 2011). The N-terminus can even engage fibril surfaces, which makes it the initiator of secondary nucleation processes (Kumari et al., 2021). It has also been reported that the functional state of αsyn could be an α-helical tetramer, also mediated by its N-terminus (Lucas and Fernández, 2020), although these findings are disputed (Binolfi et al., 2012). *In vitro* this structural change can be initiated by adding low concentrations of SDS (Chandra et al., 2003). In a pathological context, αsyn is usually N-terminally acetylated (Anderson et al., 2006), which increases its propensity for α-helices (Maltsev et al., 2012).

Familial mutations Point mutations, dupli- and triplications in the gene coding for αsyn, *SNCA*, are known to cause familial forms of PD (Nussbaum, 2018). Several known mutations that lead

to (mostly early-onset) familial PD, the first of which was even discovered before the presence of αsyn in Lewy bodies (Polymeropoulos et al., 1997), are located in the N-terminus (A18T, A29S, A30P, E46K, H50Q, G51D, A53E, A53T) (Appel-Cresswell et al., 2013; Ghosh et al., 2014; Kiely et al., 2013; Krüger et al., 1998; Kumar et al., 2018; Zarranz et al., 2004). They significantly alter the protein's properties with respect to their pathological behavior: Point mutations have been found to impact aggregation behavior (Flagmeier et al., 2016) and change the likelihood of oligomer formation (Kumar et al., 2018). Mutations as well as genetic variations of *SNCA* influence the overall clinical picture, age of onset, and severity of the disease (Corrado et al., 2018).

NAC-Region: The NAC-region, ranging from amino acid 61-90, was first found as part of amyloid deposits in Alzheimer's disease, at which time αsyn itself (together with its relative β-synuclein) was even briefly called NACP for "non-Aβ compound precursor" (Iwai et al., 1995; Uéda et al., 1993). Most of the amino acids that are buried inside the fibril core in the protein's aggregated forms due to their overall high hydrophobicity are located in the NAC-region (Rodriguez et al., 2015). This fibril core is made of varying amino acid ranges, so several polymorphs exist (Guerrero-Ferreira et al., 2020), which *in vivo* can be partially connected with different diseases (Lau et al., 2020; Liu et al., 2021) and *in vitro* with solution conditions (Sidhu et al., 2014) and post-translational modifications (Ma et al., 2016).

C-Terminus: Amino acids 91-140 are often considered as belonging to the C-terminus of αsyn. This stretch is negatively charged and more hydrophilic than the rest of the protein. Therefore, it is responsible for keeping the protein in solution and accounts for the low pI of 4.67 (Levitan et al., 2011). Accordingly, C-terminally truncated αsyn variants are known to be much more aggregation prone and also show a shifted pH range for secondary processes in low-salt regimes (but not in presence of high sodium salt concentrations), which for the wildtype are dependent on the collapse of the C-terminus due to pH- or ion-driven charge compensation (Horne et al., 2023; Ma et al., 2018; McClendon et al., 2009). The multitude of acidic residues in the C-terminus also form binding sites for divalent cations such as Ca^{2+} (Moons et al., 2020), which therefore also affect aggregation behavior (Bharathi et al., 2007). The C-terminus can also undergo posttranslational modification *in vivo*, for example, by phosphorylation (Anderson et al., 2006) but also naturally occurring truncation (Suthar and Lee, 2023).

The Physiological Role of α-synuclein

The precise function played by αsyn in the human body is not entirely understood, not due to insufficient efforts but because it seems to fulfill a number of different roles. How relevant they each are is hard to tell, since a lot of them were initially investigated just because of αsyn's implication in PD and not because αsyn's influence on certain systems was so obvious. The protein seems to be able to duplicate a lot of other proteins' functions, which in a healthy system leads to redundancy, but in the pathological state, it can influence many different sites. The list of examples below is not exhaustive.

Maybe because of its sequence homology to a family of small α-helical fatty acid-binding proteins (Clayton and George, 1998; Sharon et al., 2001), αsyn has an effect on the lipid composition of the brain, as demonstrated in *SNCA* gene-ablated mice (Barceló-Coblijn et al., 2007; Castagnet et al., 2005). It also interacts with poly-unsaturated fatty acids (PUFA), regulating the concentration of free PUFAs (Sharon et al., 2003b) but also getting oligomerized and possibly aggregated because of this interaction (Sharon et al., 2003a). Prevention of lipid oxidation is another of αsyns alleged functions (Zhu et al., 2006a), so is the reduction of brain inflammation

via modulation of Acyl-CoA synthetase (Golovko et al., 2009).
Asyn notably interferes with vesicle-trafficking pathways at the synapse by localizing at vesicles (Pranke et al., 2011) and inhibiting their mobility, thereby negatively regulating exocytosis of neurotransmitters and maintaining the homeostasis of the vesicle recycling pool (Scott and Roy, 2012). On the contrary, it also positively influences the assembly of the SNARE complex (Burré et al., 2010).
Due to its structure, αsyn has the ability to bind proteins with its N-terminus and keep the formed complex in solution with its C-terminus, granting it chaperone-like qualities (Park et al., 2002). Since it has a sequence homology to 14-3-3 proteins, which are a family of chaperones that bind kinases and positively modulate their activity (Ostrerova et al., 1999), this allows it to engage similar targets such as protein kinase C (Jin et al., 2011) and fulfill a regulating function.

The Pathological Role of α-synuclein

Mirroring the multitude of potential functions αsyn can perform, its accumulation can also cause a lot of different impairments, which are observed within a class of diseases termed synucleinopathies. These include Parkinson's Disease, but also dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA). While αsyn is likely not the only cause of these diseases, it is at least part of the pathology.

Lewy Bodies: A hallmark of PD are so-called Lewy bodies (LB), intracellular inclusions of different misfolded species of proteins. LBs can be subdivided into classical or brainstem LBs that feature a dense core and a peripheral halo and cortical LBs that lack a clear organization. Separate from these are Lewy neurites, which are dystrophic processes that contain protein accumulations and are more thread-like in shape (Braak et al., 1994). αsyn has been found to make up the bulk of proteins in LBs (Spillantini et al., 1997), partly in a phosphorylated and/or ubiquitinated state and mostly non-monomeric form (Anderson et al., 2006; Hasegawa et al., 2002). However, LBs are diverse structures that also include lipids, cytoskeletal and membraneous fragments along over 100 additional proteins (Fares et al., 2021).
It is likely that LBs can propagate, as per the observed spreading of LB pathology described by the Braak stages (see fig. 1.1). A perceived spread of LB pathology from host to grafted material was reported multiple times (Li et al., 2008). LB formation could also be induced by injecting brain homogenate from diseased animals or humans into healthy animals; however, the in-depth characterization of the LB-like aggregates was often incomplete in those studies (Masuda-Suzukake et al., 2013; Recasens et al., 2014). These findings harmonize well with the prion-like understanding of amyloid aggregation. However, the exact mechanism of propagation is still unclear. Possible explanations include simple fragmentation of material from LBs, transport in secretory vesicles (Lee et al., 2005), spreading via "nanotubes" between neurons (Agnati et al., 2010), or excretion via endosomes (Liu et al., 2009).
Even though they can be found in diseased brains, the exact role of LBs is not well understood, so little in fact that even the trajectory of their impact is not completely clear. This is in part due to the lack of animal models that exhibit Lewy pathology (Kahle, 2008). LBs are either a byproduct of processes that lead to neurodegeneration or their presence itself is detrimental. It has, for instance, been found that perhaps neither fibrillization nor LBs themselves lead to neurodegeneration but the formation of LBs (Mahul-Mellier et al., 2020). LB pathology also only correlates poorly with actual neurological decline (Parkkinen et al., 2008) as well as LBs having been found in approximately 7-18% of *post-mortem* brain analyses of people over the age of 60 without any neurological symptoms (Frigerio et al., 2011).

Dementia with Lewy Bodies PD is not the only disease that displays LB pathology. LB-like inclusions, called glial cytoplasmic inclusions, can be found in individuals suffering from multiple system atrophy, although they seem distinct in composition from real LBs (Spillantini et al., 1998). A more interesting case is the disorder known as "Dementia with Lewy bodies" (DLB) that heavily features LB pathology and is clinically nearly indistinguishable from PD. In fact, there is an ongoing debate in the field on whether DLB and PD are distinct pathologies or two slightly different phenotypes of the same disorder (Borghammer et al., 2024; Jellinger, 2024).

α-synuclein oligomers: Oligomers of αsyn can be found in *post-mortem* brains of individuals suffering from Lewy body pathology as well as in the cerebrospinal fluid of PD patients (El-Agnaf et al., 2003; Roberts et al., 2015). Autophagy is impaired in PD brains (Hou et al., 2020), which, under normal conditions, keeps the amount of oligomers and small fibrillar species in check (Klucken et al., 2012; Lu et al., 2012). Normally, microglia can uptake αsyn and degrade it; however, it has also been shown to impair microglial autophagy (Choi et al., 2020; Tu et al., 2021). The degradation of extracellular αsyn oligomers by microglia and monocytes is impaired in elderly individuals, also contributing to the risk factor of age (Bliederhaeuser et al., 2016). αsyn oligomers with differing β-sheet contents and sizes have been found, which also display different seeding and neurotoxicity properties. αsyn is mostly reported to produce primary oligomers, at least if only taking the aggregation process itself into consideration. There are both off-pathway (Zurlo et al., 2021) and on-pathway species (Cremades et al., 2012).

In vitro oligomers Oligomers that are created *in vitro* generally display a large quantity of different structures with varying properties, depending on the exact protocol (see p. 17). Here, only those that do not rely on stabilization by a chemical compound are included. *In vitro*, αsyn has been shown to shift from random coil over largely α-helical to largely β-sheet-rich structures during the process of aggregation. This confirms the existence of α-helical on-pathway oligomers, whose lifetime depends on the overall aggregation speed (Ghosh et al., 2015).
One oligomer type appears to exhibit a "hollow cylinder" morphology (Chen et al., 2015), in which the interactions of the β-sheets are mostly hydrophilic and mediated by water molecules (see fig. 1.7). This form has also been found in the disease-relevant mutants A30P and A53T,

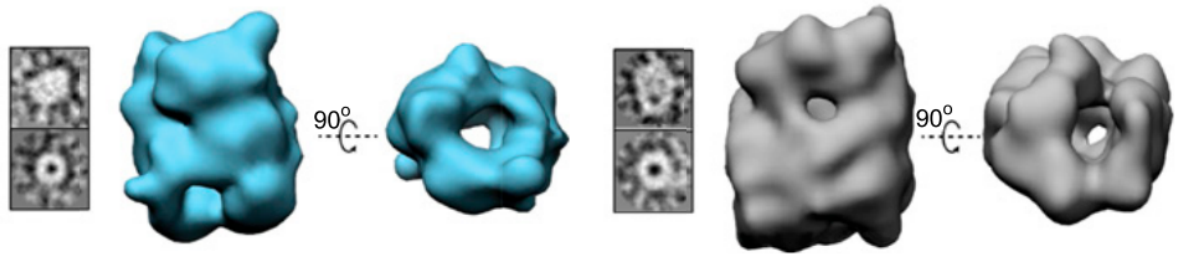

Figure 1.7: Structure of the kinetically captured αsyn oligomer formed from lyophilization. Both found populations display a "hollow cylinder" morphology. Adapted from Chen et al. (2015).

both as on-pathway oligomers, which converted into fibrils in the presence of excess αsyn monomer (Lashuel et al., 2002). This shape seems especially significant because the oligomers might form pore-like structures, which could easily disrupt the homeostasis of molecules over a membrane. In fact, a lot of negative consequences of αsyn might be explained by this property (see p. 16). Tubular oligomers were reported by the same author for the A53T variant.
Spherical oligomeric species have been reported that appear to be more stable (Cremades et al.,

2012). A convergence of smaller oligomers to larger species has also been observed, which underpins the on-pathway nature of most αsyn oligomers (Hu et al., 2016).

In vivo oligomers Resulting from the more complex systems, only a few *in vivo* oligomers of αsyn have been characterized structurally, with most studies limiting themselves to their localization, for instance, in the neuronal endoplasmic reticulum (ER) (Colla et al., 2012b), at cell membranes (Feng et al., 2010) and in lysosomes (Plotegher et al., 2014).
Pore-like αsyn oligomers have been detected *in vivo* as well as *in vitro*, which are both SDS-stable and toxic - although it should be noted that the used cell model overexpressed αsyn (Feng et al., 2010). Annular particles were reported to be released from bigger aggregates in *ex vivo* material (Pountney et al., 2004).

Toxicity: αsyn and its oligomers specifically have been shown to be neurotoxic in several models, such as rats and cell cultures of *C. elegans* and *drosophila melanogaster* (Karpinar et al., 2009; Winner et al., 2011).

Membrane disruption αsyn oligomers can disrupt membrane integrity. One proposed mechanism for this is pore formation (Volles and Lansbury, 2002), which is in line with *in vitro* and *in vivo* observed structures and leads to a loss of dopaminergic neurons (Winner et al., 2011). Another is the thinning of the membranes by rearrangement of the phospholipids, thereby altering the conductance of the membranes (Ouberai et al., 2013; Sokolov et al., 2006). A third proposition is a detergent-like mechanism which is linked to αsyns affinity for lipids (Reynolds et al., 2011), although similar effects could also be achieved by using high concentrations of monomers (van Maarschalkerweerd et al., 2014). Depending on the location and type of the membrane, this can lead to various impediments. Disruption of the cell membrane and, thereby, the calcium homeostasis can lead to the depolarization of neurons, to caspase activation, and cell death (Danzer et al., 2007). In the mitochondria, a decrease in transmembrane potential was measured after treatment with αsyn oligomers, as well as compromised cellular respiration and an increase in reactive oxygen species (ROS) (Ghio et al., 2019; Parihar et al., 2009). This not only disturbs energy production, which is especially critical in the brain, but ROS also directly lead to neurodegeneration (Patten et al., 2010).

Binding to receptors In addition to membrane-destructive behavior, αsyn oligomers have been shown to bind to a multitude of receptors. Components of the translocase of the outer membrane (**TOM**) family, which transport proteins through the outer mitochondrial membrane, can be affected by αsyn oligomers. In particular, binding to TOM20 was observed, which inhibited the protein import in PD (Di Maio et al., 2016) and a degradation of TOM40 that disrupts mitochondrial homeostasis (Vasquez et al., 2024). Mirroring one of αsyns physiological functions, its oligomers can negatively interact with the **SNARE complex** by inhibiting vesicle docking (Burré, 2015), partly via an interaction of both αsyn monomers and oligomers that leads to vesicle clustering (Choi et al., 2013; Yoo et al., 2021) and thereby inhibiting exocytosis of neurotransmitters: Oligomers of both the disease-relevant αsyn mutant A53T (Colla et al., 2012a) and wildtype αsyn can lead to ER stress (Colla et al., 2012b). In fact, another study showed that only oligomeric assemblies of αsyn could trigger the ER stress response, but not fibrils or monomers (Castillo-Carranza et al., 2012). Resulting from ER stress, the unfolded protein response can be triggered (Hrabos et al., 2024). αsyn oligomers have been shown to decrease long-term potentiation of neurons by activation of the **NMDA receptor** (Diógenes et al., 2012). They can cause neuroinflammation by binding to members of the toll-like receptor familiy (**TLR**), such as TLR4 (Hughes et al., 2019) and TLR1 and TLR2, which shifts the microglia into their pro-inflammatory form (Daniele et al., 2015; Kim et al., 2013).

Inhibition of the proteasome αsyn and especially its oligomers are implicated in a feedback loop, in which they inhibit the **proteasome** and are therefore degraded to a lesser extent. An inhibition of proteasomal activity was observed in general and specifically regarding the 20S and 26S proteasome (Emmanouilidou et al., 2010; Lindersson et al., 2004), which then led to dysfunction of the ubiquitin-proteasome system (McKinnon et al., 2020).

Neuronal signaling and synaptic impairment αsyn oligomers have a negative impact on neuronal signaling *in vivo* (Rockenstein et al., 2014). Regarding the synapse, αsyn oligomers have been shown to inhibit tubulin polymerization, decrease microtubule stability (Chen et al., 2007; Prots et al., 2013) and provoke dopamine leakage (Plotegher et al., 2017).

In vitro generation: Due to the high variability of αsyn oligomers upon chemical modification, most ways of *in vitro* generation include a **chemical compound**, a large part of which are likely involved in a redox reaction with the αsyn, for instance by oxidizing the methionines (Uversky et al., 2002; Zhou et al., 2010), two of which are located in the N- and two in the C-terminus. These include polyphenol(-)epigallocatechin gallate (EGCG) (Bieschke et al., 2010; Ehrnhoefer et al., 2008), a compound readily found in green tea which is known to pH-dependently act as both reducing and oxidizing agent (Sang et al., 2005); dopamine (Cappai et al., 2005), which is not only *the* neurotransmitter known to be heavily involved in PD but also a molecule with redox properties (Mohammad-Shiri et al., 2011); hydrogen peroxide (Zhou et al., 2010), the most straight-forward reduction agent; baicalein, a flavonoid that not only *in vivo* reduces oxidative stress (Sowndhararajan et al., 2017) but also induces oligomer-formation *in vitro* (Hong et al., 2008); 3,4-dihydroxyphenylacetic acid (Zhou et al., 2009) and even nicotine (Hong et al., 2009). Later down the line, byproducts of oxidative stress have also been shown to interact in a similar way with αsyn, such as 4-hydroxy-2-nonenal (Qin et al., 2007), acrolein (Shamoto-Nagai et al., 2007), and 4-oxo-2-nonenal (Näsström et al., 2009), which are examples of aldehydes produced during lipid peroxidation (Esterbauer et al., 1991).
An established protocol for oligomer formation without any additives (Lashuel et al., 2002) uses **lyophilization**, subsequent resuspension in high concentrations (ca. 12 mg/ml), and gel filtration. As long as the αsyn variant used in this manner contains both N- and C-terminus, this protocol is consistent albeit only yields a small amount of oligomers. It likely forms oligomers with an antiparallel intermolecular β-sheet structure, which might also form during limited hydration conditions of liquid-liquid phase separation (Camino et al., 2020; Chen et al., 2015).

Amyloid-β

Amyloid-β (Aβ) is not a protein but rather refers to a multitude of peptides ranging in size from 37-49 amino acids that are a cleavage product of the appropriately named amyloid precursor protein (APP) by β- and γ-secretase. "Aβ" normally refers to the two peptides Aβ40 and Aβ42, which are most implicated in the pathogenesis of AD. In the context of this thesis, Aβ will therefore refer to one of these two.

Structure

Aβ is normally thought of as an IDP; however, there are different studies that assign it a higher propensity for certain secondary structures: There are NMR studies that show a high propensity for an α-helical motif, however, solid-state-NMR derived simulation studies showed that this helix will readily collapse as it is only meta-stable. Other studies assign it a β-turn that encompasses the C-terminus (Sgourakis et al., 2007) or an even more differing ensemble

of conformations (Vivekanandan et al., 2011). Simulations in membrane-like surroundings indicate a shift from an α-helical regime to a β-sheeted one, which is in accordance with its high propensity for aggregation.

The Physiological Role of Aβ

The Amyloid Precursor Protein: APP is expressed in different isoforms ranging from 695-770 amino acids. It is a membrane-bound type I glycoprotein that consists of a single membrane-spanning domain, an extracellular glycosylated N-terminus containing a copper- and a heparin-binding domain as well as a Kunitz Protease Inhibitor domain and a shorter, cytoplasmic C-terminus (Kang et al., 1987) (see fig. 1.8). In humans, APP has two paralogs called APLP1 and APLP2, which seem to be functionally similar if not redundant (Heber et al., 2000; Zheng et al., 1995), but do not contain the region that includes the Aβ sequence.
Like other proteins implicated in the pathogenesis of neurodegenerative diseases, its function is not completely clear. However, it has been implicated in the regulation of neurogenesis (Trapp and Hauer, 1994). Specifically, full-length APP has been shown to affect neural migration (Young-Pearse et al., 2007), axonal outgrowth (Southam et al., 2019) and synapse formation (Hoe et al., 2012).

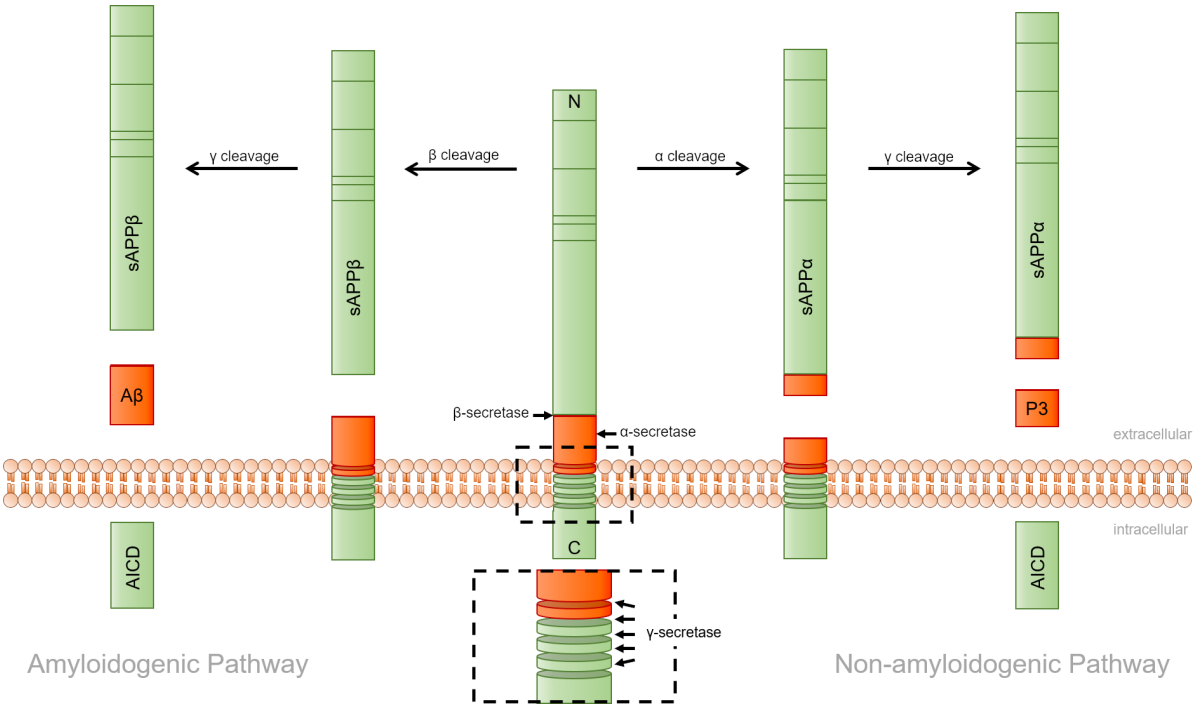

Figure 1.8: The structure of the amyloid precursor protein (middle) and its cleavage products on the amyloidogenic (left) and non-amyloidogenic (right) pathway. The Aβ sequence is highlighted in orange.

APP has multiple cleavage sites that enable endoproteolysis by α-, β-, and γ-secretases (see fig. 1.8). It can be initially cleaved by α- or β-secretase in the ectodomain, generating α- or β-C terminal fragments which are still tethered to the membrane. The α-secretase cleaves within the Aβ-sequence, thereby halting Aβ-generation and initializing the therefore so-called non-amyloidogenic pathway; whereas the β-secretase cleaves off the N-terminus in front of the Aβ-sequence, starting the fragment on the amyloidogenic pathway. The γ-secretase cuts at multiple possible sites in the membrane domain, thus yielding the many different lengths of

fragments and also releasing them from the membrane (Nunan and Small, 2000). Aβ40 and Aβ42 are therefore created by cleavage of both β- and γ-secretase.

Non-amyloidogenic APP cleavage products: The other cleavage products are less involved in AD pathogenesis, but mostly fulfill regulatory and protective functions.

Soluble secreted APP α and β (sAPPα and sAPPβ) sAPPα is the part of the ectodomain that gets cleaved by α-secretase on the non-amyloidogenic pathway and is thereby secreted into the extracellular lumen. It has been shown to have neuroprotective properties (Copanaki et al., 2010; Furukawa et al., 1996a) and seems to be the most responsible for the impact of APP on neural plasticity (Ring et al., 2007). However, it has also been found to act as a neurotoxin (Barger and Harmon, 1997). sAPPβ is the equivalent peptide that is produced by extracellular cleavage on the amyloidogenic pathway. It displays similar properties to sAPPα, although it is far less potent in its neuroprotective role (Furukawa et al., 1996b).

The APP intracellular domain (AICD) AICD is released into the cytosol after cleavage by the γ-secretase and does not contain parts of the Aβ-sequence. It has regulatory functions in gene and ER-mitochondria signaling and is involved in correct neuronal function. It also inhibits neurogenesis in adult brains by suppressing the proliferation of neural precursor cells and instead promotes neurite outgrowth as well as synaptogenesis (Ng et al., 2024).
However, AICD has also been implicated in neurodegeneration, as it has been shown to modulate neuronal apoptosis and neuroinflammation (Ghosal et al., 2010; Nakayama et al., 2008), which is in line with its levels being elevated in AD patients (Ghosal et al., 2009).

The p3 peptide (P3) The p3 peptide is a truncated 3 kDa form of Aβ, namely amino acids 17-42, which is produced on the non-amyloidogenic pathway, but has antithetically also been found in the brains of AD and Down syndrome patients (Lalowski et al., 1996). It has also been shown to exhibit neurotoxic capabilities lower than Aβ42 and has therefore seldom been the focus of study as opposed to full-length Aβ (Ando et al., 2001).

The Pathological Role of Aβ

Extracellular Plaques: The most prominent hallmark of AD are extracellular amyloid plaques, which were first identified in post-mortem brains of AD patients (Masters et al., 2015). Their spread follows a pattern that is reproducible enough to have resulted in a staging scheme (from entorhinal/perirhinal Cortex over the Ammon subfields and the association Cortex to the Primary neocortex) for the disease, termed “Braak stages“ after its first observers (Braak and Braak, 1991; Thal et al., 2002). The structure of these plaques seems to be unique to AD, as they consist of an Aβ-containing fibrillar core surrounded by tau neurofibrillary pathology, as opposed to lesions found for instance in *dementia pugilistica* or other traumatic brain injuries (Nelson et al., 2009). The Aβ can either be found in its original form, modified or N-terminally truncated (Portelius et al., 2010) and forms distinguistable superstructures (Han et al., 2017).
Histologically, amyloid plaques are often surrounded by altered neuronal activity (Spires-Jones and Hyman, 2014), however, the correlation of Aβ plaque load to the extent of cognitive decline is far less pronounced than for tau NFTs (Nelson et al., 2009). This, in part, informs the shift away from fibrils to oligomers as a focus of investigation, which will be examined separately.

Intracellular Aβ: Another factor in the pathological effects of Aβ is linked to its localization (LaFerla et al., 2007). Even though APP can also be found in the trans-Golgi network (Xu et al., 1995), the ER and other membranes, such as endosomal and lysosomal (Kinoshita et al., 2003)

as well as mitochondrial (Mizuguchi et al., 1992), most of it is localized in the cell membrane. Due to the distribution of cleavage sites, the γ-secretase releases the Aβ into the extracellular space and most of the intracellular found Aβ has been internalized via endocytosis, not produced intracellularly (Koo and Squazzo, 1994). Out of the different types, Aβ42 is most often found intracellularly (Gouras et al., 2000). The potential for internal production should be kept in mind, however, since APP overexpression (Rovelet-Lecrux et al., 2006), e.g., in mouse models, and certain mutations of APP, e.g., APP_{SWE} (Martin et al., 1995), can upregulate it. Also, neurons alone seem to exhibit at least some production of Aβ42 in the ER (Cook et al., 1997) and possibly Aβ40 in the trans-Golgi network (Hartmann et al., 1997).
Apart from endocytosis, Aβ can be internalized via a number of receptors, as is the case for Prp^c (Foley et al., 2020), α7nAChR (Ma and Qian, 2019), apolipoprotein E (APOE) and members of the low-density lipoprotein receptor (LDLR) family (Bu et al., 2006).
Some pathways of Aβ internalization in, e.g., glial cells, might fulfill a regulatory role in preventing the rise of concentration of extracellular species, however, neuronal intake and subsequent intracellular accumulation of Aβ is an early pathological marker of AD (LaFerla et al., 2007). Studies show that Aβ oligomerization occurs more often in cells (Walsh et al., 2000), specifically in the endo-lysosomal system (Takahashi et al., 2004). This is likely due to factors such as an acidic milieu (see p. 27) and the presence of lipid membrane systems (Waschuk et al., 2001).
In turn, it has been shown that intracellular Aβ species can spread via cell lysis (D'Andrea et al., 2001) and seed extracellular amyloid plaques (Hu et al., 2009).

Aβ oligomers

Similar to α-syn, Aβ oligomeric species display a heterogeneous size distribution ranging from small di- or trimers to sizes which begin overlapping with fibrils (Ehrnhoefer et al., 2008; Glabe, 2008). Most aggregated species and thus also oligomers are formed by Aβ42, not Aβ40, likely due to the increased hydrophobicity (Jarrett et al., 1993; Lee et al., 2011). Aβ oligomers can be formed by primary and secondary nucleation (Cohen et al., 2013; Sahoo et al., 2020) and can be both on- and off-pathway (Cohen et al., 2013; Gao et al., 2020). They show a mixture of beta-sheet (parallel and anti-parallel (Cerf et al., 2009; Chimon et al., 2007) up to a cross-β structure (Stroud et al., 2012) have been reported) and coil-content, dependent on the solution and investigation conditions (Shea and Daggett, 2022). However, they seem to have a core that is resistant to, for instance, hydrogen exchange and consists mostly of beta-sheets (Serra-Vidal et al., 2014).

Toxicity of Aβ oligomers: Aβ oligomers are generally thought of as the toxic species among the different assemblies of Aβ. (Huang and Liu, 2020) Much more than α-syn, the neurotoxicity of Aβ is often mediated by different receptor-proteins to which the oligomers can bind ($P75^{NRT}$ (Knowles et al., 2009; Murphy et al., 2015; Shen et al., 2019), LRP (Deane et al., 2008; Donahue et al., 2006), PrP^c (Foley et al., 2020), mGluR5 (Brody and Strittmatter, 2018; Chen et al., 2023; Renner et al., 2010), α7nAChR (Arévalo-Serrano et al., 2008; Ma and Qian, 2019), NMDAR (Liu et al., 2019; Tackenberg et al., 2013), β-AR (Gibbs et al., 2010; Jin et al., 2023), PirB (Kawaguchi et al., 2022), TLR4 (Hughes et al., 2020) and FcγRIIb (Kam et al., 2013)).
This can start neurotoxic signaling cascades resulting in calcium influx (e.g., β-AR (Busche et al., 2008) and NMDA (Talantova et al., 2013)), lead to cell death (e.g., $P75^{NRT}$ (Knowles et al., 2009)), inhibit long-term potentiation (e.g., TLR4 (Hughes et al., 2020)), or cause ER stress (e.g., FcγRIIb (Kam et al., 2013)) (Umeda et al., 2011). Accumulation of Aβ has also been found to inhibit long-time potentiation in tandem with tau protein (Chen et al., 2000; Shipton et al., 2011;

Wang et al., 2002) and lead to mitochondrial dysfunction (Kaminsky et al., 2015) of respiratory chain complexes III and IV (Caspersen et al., 2005). Aβ oligomers can stimulate microglia and lead to their death (Maezawa et al., 2011). In other cases, the binding causes inflammation (e.g., TLR4) and thus contributes to neuronal damage, a property often ascribed to protofibrils or oligomers at later stages. (Chakrabarty et al., 2018; De et al., 2019; Reed-Geaghan et al., 2009; Udan et al., 2008) Aβ42 Oligomers can also induce lipid membrane permeability (Flagmeier et al., 2017), while monomers and fibrils cannot. While this property seems to be size- and hydrophobicity-dependent (Mannini et al., 2014), there are conflicting reports in literature on whether earlier species (De et al., 2019) or protofibrils (Yasumoto et al., 2019) are more involved. As with other properties, the remark has to be made that Aβ oligomers from different sources show vastly different destructive potential, which explains the general shift of the field towards *ex vivo* material (Al Adem and Lee, 2023).

Tau missorting: A detrimental effect that should be highlighted in the context of this thesis, is the interplay of Aβ oligomers with the protein tau, which is called "tau missorting": Human tau is mainly expressed in neurons and is associated with microtubules by stabilizing tubulin polymerization. It is a protein ranging from 352 to 441 amino acids in size due to many different isoforms that stem from alternative splicing (Goedert et al., 1989). As an IDP, it lacks a fixed secondary structure, but can be divided into two distinct regions: The projection and the microtubule-binding domain containing multiple repeat domains (Mukrasch et al., 2009). The repeat domains in their hyperphosphorylated form are also the main component of the paired helical fragments (PHFs), fibrillar structures that, in turn, form the NFTs found in AD (Serrano-Pozo et al., 2011). The intracellular distribution of tau changes along neuronal maturation: During embryonic development, where it is generally upregulated (Drubin et al., 1984), it is evenly distributed in the cell body and neurites but becomes sorted into the axons in mature neurons (Mandell and Banker, 1995). In AD, tau becomes "missorted" into the somatodendritic compartment in the early stages of the disease before forming NFTs. Since NFT load correlates well with cognitive decline, this makes tau and specifically tau missorting, which can also be triggered by Aβ oligomers (Zempel et al., 2010), a critical research target in the study of AD (Braak and Del Tredici, 2004).

DimAβ as an oligomer model: Two key challenges in investigating amyloid oligomers are for one, the dependency on protein concentration and, secondly, the short half-life. This is especially true for proteins with a high aggregation propensity such as Aβ42, since the population density of oligomers is at its height before and at the onset of aggregation. The shorter this transition phase, the shorter the opportunity for measurements. The isolation of particular species is hard as well, since all amyloid species are in equilibrium with each other, and a dilution of oligomers may well lead to their dissolving since their formation is concentration-dependent.
For Aβ42, in particular, a very high concentration ($> 50\ \mu$M) is needed to induce oligomerization without any chemical additives or crowding agents (Fu et al., 2015). In a ThT-assay, this takes the form of a biphasic kinetic profile with a lag-free first phase corresponding to oligomerization and a second aggregation phase. The Hoyer lab has developed a head-to-tail dimer of Aβ40, called dimAβ for short, that is covalently linked with a flexible linker to mimic a higher concentration without the drawbacks of such (Hasecke et al., 2018). Due to being in close proximity to each other, the two Aβ40-subunits form oligomers at lower concentrations, which makes experimentation more advantageous. DimAβ oligomers have a very characteristic, curvilinear shape that corresponds to a beads-on-a-chain structure (see fig. 1.9).
DimAβ oligomers are more kinetically stable than Aβ oligomers, off-pathway and therefore

inhibit aggregation, which in combination leads to a separation of both kinetic phases. The flexible glycine-serine-linker is 20 amino acids long and has been shown to neither interfere with the disordered conformation of the monomer nor to incorporate into the aggregated forms via NMR experiments.

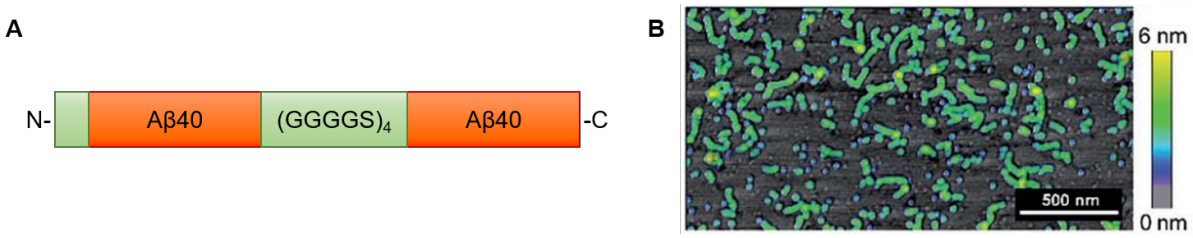

Figure 1.9: The synthetic Aβ dimer dimAβ. (A) The composition of the dimer. A methionine was added in the beginning to facilitate expression. (B) The typical curvilinear structure of dimAβ oligomers as seen under the AFM. Adapted from Hasecke et al. (2018).

A dimer of Aβ42-subunits also exists, though in contrast to dimAβ, the linker seems to be involved in its oligomerization which hinders its usefulness as a model protein. Despite this, it forms similar curvilinear oligomers and fibrils (Unpublished data).

Who shot first: Conflicting Triggers for AD

The Aβ peptide and its deposition are most frequently mentioned as the initial pathological event in AD. The hypothesis devised around this peptide and its many forms is called the "amyloid cascade hypothesis" (ACH) and most prominently competes with an alternative featuring the protein tau as an initiator. While there is no denying that Aβ is *somehow* implicated in the pathogenesis of AD, for therapeutic reasons, it is essential to determine the distinct starting point - and not just a potentially secondary marker of AD.

The amyloid cascade hypothesis In 1992, Hardy and Higgins proposed the ACH, which posits that Aβ is the causative agent of AD pathology (Hardy and Higgins, 1992). They state that "Aβ[...] itself or APP cleavage products containing the Aβ[-sequence] are neurotoxic and lead to neurofibrillary tangle formation and cell death". They explicitly claim that 1. tau NFTs might be a consequence of Aβ disrupting calcium homeostasis, and therefore both acknowledge its involvement and cement its role as a downstream effect rather than a cause of AD; and 2. that there is a relationship between plaques, NFTs, and clinical dementia. Until then, it has evolved, for instance, to include the previously mentioned proposed consequences of (oligomeric) Aβ accumulation in the brain, but it remains a linear path from APP cleavage to dementia (Selkoe, 2000).

Observations refuting the ACH Traumatic brain injury (TBI) is a risk factor for AD and is known to cause APP to be transported to the damaged sites (Hortobágyi et al., 2007). Accordingly, it also causes an increase in Aβ oligomers (Washington et al., 2014). Both observations can be interpreted as indicating not only that APP is vital in the repair of nerve cells, but also that neuronal injury precedes Aβ pathology and also potentially tau pathology (Irving et al., 1996). There is a long latency period between first appearances of Aβ pathology and clinical dementia. In fact, individuals can carry substantial amyloid burden without showing any symptoms for years (Aizenstein et al., 2008; Villemagne et al., 2011). This raises the question of whether amyloid is sufficient to cause AD. Mouse studies do not disperse this doubt, since transgenic

mouse models that express human APP constructs do not reproduce the full clinical picture of AD (they show synaptic loss but no neurodegeneration or NFT pathology, for instance), and even then, most of them overexpress a fAD variant of APP at superphysiological levels (Elder et al., 2010).
Mice can be cured from their amyloid-induced state, humans cannot, as of yet. One of the biggest detractors of the ACH, is therefore the lackluster outcome of clinical studies testing Aβ-targeting drugs (see p. 23), which even if they succeeded in decreasing amyloid burden, failed at improving cognition.

The Danger of hyperfocusing on the ACH The ACH is not the only model that tries to explain the pathogenesis of AD, but it is by far the most centered in AD research (see fig. 1.10). This leads to a concentration of resources and interest that pushes other scientific avenues aside, hindering the investigation of alternative theories. Among these are failing autophagy (Qian 2017), failing lysosomal function (Nixon and Cataldo, 2006), mitochondrial dysfunction (Sharma et al., 2021), aberrant cell cycle control (Arendt et al., 2010; McShea et al., 1997), progressive oxidative damage (Zhu et al., 2006b), and neuroinflammation (Twarowski and Herbet, 2023). This list is not exhaustive; the biology of AD pathology is complex and trying to force every observation into the context of the ACH risks misinterpretation.

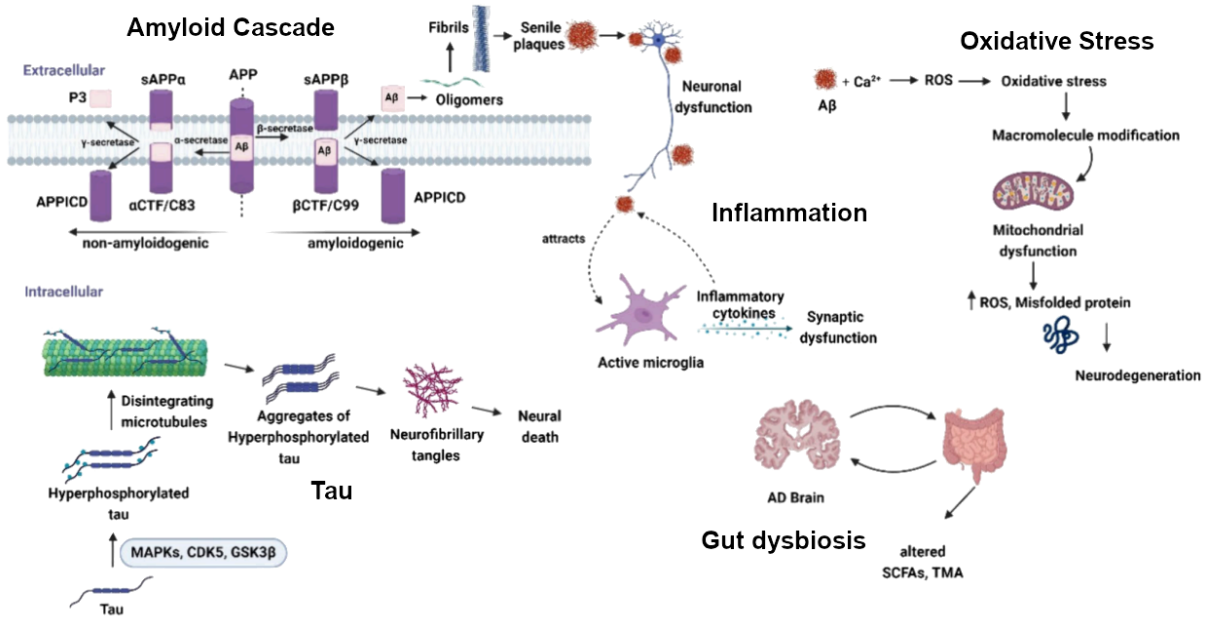

Figure 1.10: The multitude of competing hypotheses of the pathogenesis of AD (apart from the ACH). Adapted from Reddi Sree et al. (2025).

Therapeutic Approaches for Amyloid Diseases

A general requirement for all pharmacological approaches towards neurodegenerative amyloid diseases is the need to cross the blood-brain barrier (BBB) in impactful amounts. Given this property, pharmaceuticals that aim to have a lasting effect need to focus not on the symptoms but on the underlying pathologies, and only therapeutic approaches that satisfy this condition will be discussed here.

Alzheimer's Disease

Anti-inflammatory drugs A potential avenue for therapeutic potential are anti-inflammatory drugs that have the ability to cross the BBB. Promising candidates are members of the tetracycline family of antibiotics, which are suspected to have anti-amyloidogenic properties (Markulin et al., 2022; Rahmani et al., 2022). In mice, they have been additionally shown to counteract both Aβ synthesis and tau hyperphosphorylation, as well as to improve cognition (Gomez-Murcia et al., 2022; Zhao et al., 2022). While some of these drugs are already approved for usage in humans, clinical trials that test the efficacy of their anti-amyloidogenic properties have yet to be conducted.

α-, β- and γ- secretase modulators Since APP is either processed via the amyloidogenic or the non-amyloidogenic pathway, increasing the activity of α-secretase inhibits the synthesis of Aβ by depleting APP. Multiple α-secretase modulators have been studied in Phase II clinical trials, but none has progressed into Phase III as of yet (Cummings et al., 2019; Vellas et al., 2011). The more straightforward approach is to directly inhibit the β-secretase, and this has been a popular scientific endeavor. However, not only did multiple such inhibitors fail in Phase II due to toxicity in humans, some of the ones that passed showed no cognitive improvement even when lowering Aβ levels in the CSF, and some even allowed for cognitive worsening (Bazzari and Bazzari, 2022; Fish et al., 2019). The attempts to inhibit γ-secretase have fared even worse, which is likely due to unspecifity, as it is not only involved in both pathways but also cleaves a large variety of other transmembrane proteins, such as the Notch receptor (Cummings et al., 2019; Zhou et al., 2022). Multiple clinical trials had to be discontinued or terminated due to adverse effects whilst displaying limited efficacy (Coric et al., 2015; Doody et al., 2013).

Aggregation modulators Most compounds that try to interfere with Aβ aggregation are **monoclonal antibodies** designed to bind the monomer and eliminate it before it can aggregate into larger species. Seven of these have advanced into Phase III; however, those that have not been aborted because of missing clinical benefits show unsatisfactory results (Reddi Sree et al., 2025). In 2021, the antibody Aducanumab was approved by the FDA but not by the EMA, citing no proven efficacy and brain swelling as an adverse effect, and has since been discontinued. In 2024, Donanemab was approved by the FDA and has applied for approval in the EU, but the efficacy of this antibody is also debated (Doggrell, 2021).
Due to the interaction of Aβ with metal ions and subsequent aggregation, metal protein attenuating compounds (**MPACs**), such as clioquinol, have been investigated and have been found to maintain synaptic health in both mice and neuronal cell culture and decrease Aβ levels in the blood (Perez et al., 2019). However, long-term safety of these drugs is debated.
Another intervention, which is being investigated and that has been increasingly in the spotlight, are **D-enantiomeric peptides** that interact with either Aβ oligomers or hyperphosphorylated tau. Since physiological amino acids are L-enantiomeric only, the half-life of these peptides is greatly increased. D-peptides against Aβ have shown enhanced cognition in beagle dogs and have been approved for a Phase II trial by EMA under the name PRI-002 (Kutzsche et al., 2020, 2023). D-peptides have also been positively tested against tau NFT formation in mouse models, but have not yet progressed to human trials (Hou et al., 2024).

Vaccinations Since plaque build-up far precedes the occurrence of clinical symptoms in AD, the idea for a vaccination against either Aβ or hyperphosphorylated tau is self-evident. However, as of now, only one candidate for Aβ has passed a Phase II trial, called ABvac40, a monoclonal antibody that focuses on the C-terminal segment of Aβ40 (Lacosta et al., 2018). More promising results were achieved for an anti-tau antibody namely AADvac1, which has not only shown

good tolerability but has also passed a Phase II clinical trial while showing antibody-dependent slowing of AD-related decline (Cullen et al., 2024).

Parkinson's Disease

αsyn aggregation inhibitors Although it is not yet completely clear which aggregated form of αsyn is responsible for the majority of damage done in PD, pharmaceuticals targeting the protein are being investigated. One such compound is called NPT-100-18A, a **peptidomimetic** discovered through structure-based drug discovery, which not only interferes with αsyn's ability to interact with membranes but also, more importantly, inhibits dimerization and oligomer formation (Wrasidlo et al., 2016). It was shown to decrease both mitochondrial oxidative stress and neuronal degeneration (Alecu et al., 2025). The second-generation compound is currently in preparation for Phase II under the name UCB0599 (Price et al., 2018; Smit et al., 2022). Small **aminosterols** derived from the dogfish shark have been found to displace αsyn oligomers from membranes and have been proposed to inhibit lipid-induced primary nucleation. One, called squalamine, has completed a Phase II clinical trial under the name ENT-01 (Camilleri et al., 2022). As with Aβ, there have been clinical trials for **monoclonal antibodies** as well, one of the most advanced being prasinezumab, which is currently in Phase II (Pagano et al., 2024). An **active immunotherapy** mimicking αsyn epitopes has completed Phase I (Volc et al., 2020).

Stearoyl-CoA desaturase inhibitors Since αsyn likely aggregates at interfaces and has a high affinity for lipid membranes, targeting the composition of the membrane is another starting point for interfering with this binding (Fanning et al., 2022). Inhibitors of the enzyme stearoyl-CoA desaturase (SCD), which catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids, have proven to prevent PD phenotypes in mouse models and have recently cleared a Phase Ib trial under the name NL9172 (Nuber et al., 2021).

Glucocerebrosidase agonists One of the families of genes that have been implicated in familial PD forms is that of the *GBA* genes, of which *GBA 1* encodes the lysosomal enzyme glucosylceramidase β. In fact, a mutation of this gene is one of the highest known genetic risk factors (Schapira, 2015). Since the mutation decreases the expression levels of the enzyme, and low enzyme concentration leads to αsyn accumulation, compounds that either elevate the level or enhance the enzyme's effects are a logical intervention. One such glucocerebrosidase activator called LTI-291 has recently passed a Phase Ib trial and was well-tolerated; another, called Ambroxol, has recently started Phase III (Colucci et al., 2023; den Heijer et al., 2023).

LRRK2 inhibitors Another gene implicated in familial PD is *LRRK2*, encoding the leucine-rich repeat kinase 2 that is hyperactive in mutated forms. LRRK2 phosphorylates a subgroup of G-proteins called RABs, which are implicated in the regulation of vesicular transport and regulate their efficacy (Alessi and Sammler, 2018). LRRK inhibitors are therefore a try to bring the expression back to baseline, and one such compound has recently been tested in a Phase I clinical trial under the name DNL201 (Jennings et al., 2022).

Aim of the Study

Amyloid oligomers of Aβ and αsyn are implied in the pathogenesis of AD and PD, respectively. However, their innate transience hampers the investigation of their effects on relevant biological systems and processes. The synthetic Aβ40 dimer dimAβ has the advantage of kinetic stabilization, which makes it a prime tool for both *in vitro* and *in vivo* research, allowing for longer experimental setups and better separation of parallel aggregation processes.

In the first chapter, dimAβ is applied to investigate the discrepancy between the many *in vivo* studies that show the large significance of Aβ oligomers in pathological processes and the fact that *in vitro* studies need considerably higher concentrations to replicate the structures observed *in vivo*. It raises the question of how aggregation data acquired *in vitro* can realistically be translated into knowledge gain, contributing towards the understanding of pathological processes if the mechanism is not securely the same. This difference in needed to present concentration is one of the most significant points of contention in the connection of results acquired in different experimental setups. We find that reducing the pH to values found in the endo/lysosomal system not only drastically increases the oligomerization rate but also decreases the needed concentration into a range that has previously been measured in this cellular compartment. In the same study, we investigate the effect of dimAβ on primary mouse neurons and are able to replicate more behaviors of Aβ oligomers, thereby confirming the plausibility of using dimAβ instead of Aβ without losing significance.
In the second chapter, dimAβ is applied to investigate the effect of Aβ oligomers on the secondary nucleation of αsyn. This process and its potential for exponential fibril proliferation is not only a central step in amyloid aggregation but has also been hypothesized to contribute to the spread of amyloid pathology. Considering that deposits of Aβ have also been observed in PD, the cooperative behavior of these two proteins is of interest. In addition to this, *in vivo* aggregation processes occur in the presence of many more molecules than in controlled *in vitro* settings. Therefore dimAβ is also used to test for potential hetero-inhibition of secondary nucleation to find out if proteins that do not share the sequence of the fibril-proteins, can inhibit this process.

2

MANUSCRIPTS

2.1. Schützmann*, Hasecke*, Bachmann*, Zielinski, Hänsch, Schröder, Zempel & Hoyer (2021) Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce Tau missorting, *Nat Commun* 12, 4634.

Contribution (Shared First Author*): Expression and purification of dimAβ, design of ThT experiments, ThT assays, fitting of kinetic curves, AFM imaging, cell culture experiments for colocalization of dimAβ and lysosomes, preparation of figures, writing parts of the initial draft, reviewing the manuscript

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Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce Tau missorting

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Amyloid-β peptide (Aβ) forms metastable oligomers >50 kDa, termed AβOs, that are more effective than Aβ amyloid fibrils at triggering Alzheimer's disease-related processes such as synaptic dysfunction and Tau pathology, including Tau mislocalization. In neurons, Aβ accumulates in endo-lysosomal vesicles at low pH. Here, we show that the rate of AβO assembly is accelerated 8,000-fold upon pH reduction from extracellular to endo-lysosomal pH, at the expense of amyloid fibril formation. The pH-induced promotion of AβO formation and the high endo-lysosomal Aβ concentration together enable extensive AβO formation of Aβ42 under physiological conditions. Exploiting the enhanced AβO formation of the dimeric Aβ variant dimAβ we furthermore demonstrate targeting of AβOs to dendritic spines, potent induction of Tau missorting, a key factor in tauopathies, and impaired neuronal activity. The results suggest that the endosomal/lysosomal system is a major site for the assembly of pathomechanistically relevant AβOs.

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Aβ amyloid fibrils are highly stable protein aggregates of regular cross-β structure that constitute the main component of the senile plaques in the brains of Alzheimer's disease (AD) patients[1–3]. Although amyloid fibrils can exert toxic activities, metastable Aβ oligomers are thought to represent the main toxic species in AD[3–5]. At sufficiently high monomer concentration, Aβ readily forms oligomers with molecular weights (MWs) >50 kDa with spherical, curvilinear, and annular shapes, where the elongated structures appear as "beads-on-a-string"-like assemblies of spherical oligomers[4–11]. While multiple names have been given to these metastable Aβ oligomers, including AβOs, ADDLs, and protofibrils, they seem to be closely related with regard to their structures and detrimental activities and likely form along a common pathway[6,7,12]. Importantly, this pathway is distinct from that of amyloid fibril formation, i.e., AβOs are not intermediates on the pathway to amyloid fibrils (they are "off-pathway") but constitute an alternative Aβ assembly type with distinct toxic activities (Fig. 1a)[4,5,11,13]. The distinct nature of Aβ amyloid fibrils and AβOs is also reflected in their different formation kinetics. Aβ amyloid fibrils form by nucleated polymerization with crucial contributions from secondary nucleation processes, resulting in the characteristic sigmoidal growth time courses that feature an extended lag time[14]. AβOs, on the other hand, form in a lag-free oligomerization reaction that has a substantially higher monomer concentration dependence than amyloid fibril formation[11]. We note that in this work the term AβO refers exclusively to these off-pathway oligomers and does not include other oligomeric Aβ species, such as those transiently formed on the pathway to amyloid fibrils, through secondary nucleation, or through shedding by fibril fragmentation[15].

Several lines of evidence support a critical role of AβOs in AD pathogenesis. AβOs of sizes >50 kDa are the main soluble Aβ species in biological samples[16]. They are synaptotoxic, disrupt long-term potentiation, and cause cognitive impairment in mouse and non-human primate models[4,8,17–23]. Furthermore, AβOs induce oxidative stress, endoplasmic reticulum stress, neuroinflammation, and elicit Tau missorting, the earliest hallmark of tauopathy in AD[21,23–29]. The detrimental effects are enhanced by pathogenic Aβ mutations that specifically promote AβO formation, in particular the arctic (Aβ E22G) and the Osaka (Aβ ΔE22) mutations[22,23,28,30,31]. Consequently, targeting AβOs therapeutically is an important alternative to amyloid-centric approaches and has entered clinical evaluation[32–34].

AβOs were suggested to trigger toxic effects through ligand-like binding to a remarkably high number of candidate receptors[4,35]. AβOs achieve clustering of receptors in cell surface signaling platforms, probably promoted by the multivalency inherent to AβOs[4,35,36]. AβO clustering is especially prominent at dendritic spines, which deteriorate upon prolonged exposure to AβOs[18]. Importantly, this effect is mediated by Tau protein, providing a connection between the Aβ and the Tau aspects of AD pathogenesis. AβOs induce missorting of Tau into the somatodendritic compartment as well as Tau hyperphosphorylation, leading to microtubule destabilization and spine loss[23,37–39].

In addition to receptor binding of extracellular AβOs, intracellular AβOs are thought to contribute to AD pathogenesis[40]. The endosomal–lysosomal system is the main site not only for Aβ production but also for the uptake of Aβ monomers and AβOs[27,41–49]. Aβ accumulates in endosomes/lysosomes, which promotes aggregation with potential consequences for cellular homeostasis as well as for the spreading of Aβ pathology by exocytosis of aggregated Aβ species[27,28,41,44–46,48–51].

At neutral pH, high Aβ concentrations are required to convert a substantial fraction of the protein into AβOs. Widely used protocols for AβO preparation start from around 100 µM Aβ

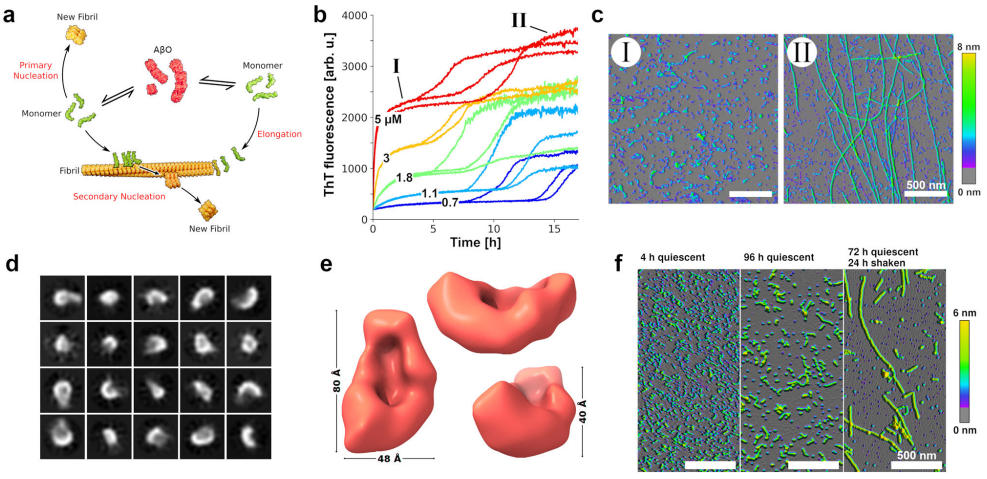

Fig. 1 AβOs assemble from dimAβ in a lag-free oligomerization reaction. a Scheme of AβO and amyloid fibril formation. **b** Biphasic assembly kinetics of dimAβ at pH 7.4 and indicated concentrations monitored by ThT fluorescence. The experimental replicates illustrate the good reproducibility of the nucleation-free oligomerization phase and the stochastic nature of the nucleation-dependent fibril growth phase. **c** AFM images corresponding to the two kinetic phases as indicated in **b**. **d** Exemplary 2D classes of the smallest dimAβ AβO species observed in cryo-EM micrographs. **e** 3D density reconstruction of this dimAβ AβO species at a resolution of 17 Å by cryo-EM. The comparatively low resolution is due to the small size and high degree of heterogeneity of the dimAβ AβO species. Consequently, only a rough estimate to size and volume can be made. **f** AFM images of dimAβ assemblies formed upon incubation at pH 7.4 in microcentrifuge tubes. Kinetics data as shown in **b** was obtained from at least three independently prepared assays with two to three replicates for each concentration for reproducibility. AFM images in **c** were prepared from two independent assays and at least three areas at different positions on the mica surface were scanned. The experiment in **f** was done once and at least two sections of the mica surface were scanned.

monomers[7,8,10]. At tenfold lower Aβ concentration, the formation of AβOs is already greatly disfavored, which enables the investigation of the pure sigmoidal time course of amyloid fibril formation, including the analysis of on-pathway oligomer formation[14,15,52]. These on-pathway oligomers, however, are short-lived, rapidly consumed in the process of fibril formation, and, as evident from the different assembly kinetics, clearly distinct from the neurotoxic off-pathway AβOs introduced above. To investigate AβO formation, we have generated a dimeric variant of Aβ termed dimAβ, in which two Aβ40 units are linked in one polypeptide chain through a flexible glycine–serine-rich linker[11]. In dimAβ, the conformational properties of the Aβ40 units are not altered as compared to free Aβ40 monomers[11]. The linkage of two Aβ units, however, increases the local Aβ concentration, which strongly promotes the highly concentration-dependent formation of AβOs[11] (Fig. 1b, c). The advantages in applying dimAβ for the study of AβOs are: First, AβOs form already above a threshold concentration (critical oligomer concentration (COC)) of ~1.5 μM dimAβ at neutral pH. Second, the increased local Aβ concentration preferentially accelerates AβO formation as compared to Aβ fibril formation, resulting in an enhanced separation of the kinetic phases of AβO and Aβ fibril formation, which facilitates analysis.

There is an apparent discrepancy between the obvious pathogenic relevance of AβOs and the high μM Aβ concentrations required for the conversion of a substantial fraction of the protein into AβOs at neutral pH in vitro, which exceeds the estimated picomolar to nanomolar concentrations of extracellular Aβ in normal brain by several orders of magnitude[44]. However, accumulation of Aβ in the endo-lysosomal system was shown to result in micromolar Aβ concentrations in late endosomes and lysosomes[44], suggesting that these acidic vesicles might be the prime sites of AβO formation. Acidic conditions have been reported to accelerate Aβ aggregation[53]. Here we applied dimAβ and Aβ42 to test whether pH reduction from neutral to endo-lysosomal pH affects AβO formation. We find that endo-lysosomal pH in fact strongly accelerates AβO formation, whereas amyloid fibril formation is delayed, suggesting that AβO formation is the dominant aggregation process in endosomes/lysosomes. We furthermore show that dimAβ is a disease-relevant model construct for pathogenic AβO formation by demonstrating that dimAβ AβOs target dendritic spines, induce AD-like somatodendritic Tau missorting, and reduce synaptic transmission in terminally matured primary neurons. This indicates that dimAβ-derived oligomers are suitable for the study of downstream mechanistic and neuropathological events in the progression of AD.

Results

DimAβ assembles into AβOs that bind to dendritic spines and potently induce Tau missorting. The assembly kinetics of dimAβ at neutral pH monitored by ThT show a biphasic behavior above a concentration (COC) of ~1.5 μM, with the first phase corresponding to the lag-free oligomerization into AβOs and the second phase reflecting amyloid fibril formation[11] (Fig. 1b, c). DimAβ AβOs are of spherical and curvilinear shape (Fig. 1c) and rich in β-structure[11], in agreement with the characteristics of AβOs formed from Aβ40 and Aβ42 (refs. [4–6,9,13,21]; for atomic force microscopic (AFM) data of AβOs formed from Aβ42, see below). We applied cryogenic electron microscopy (cryo-EM) to further characterize dimAβ AβOs structurally. Structure determination is hampered by the size and shape heterogeneity of AβOs[7,9,10], which is moreover evolving with time, as observed for AβOs formed from Aβ[9] as well as dimAβ[11]. As larger AβOs seem to be assemblies of small spherical structures, our analysis focused on the small AβOs observed in the micrographs (Fig. 1d, e and Supplementary Figs. 1–3). The fraction of small AβOs was 72 ± 12% in terms of particle number but only ~2–3% in terms of the number of Aβ molecules within AβOs (Supplementary Fig. 1c). The relation between the small and the elongated curvilinear AβOs cannot be inferred from the micrographs. Nevertheless, structure elucidation of the small AβOs could provide insight into a biologically relevant AβO substructure that may furthermore laterally associate and convert into protofibrillar AβOs[54]. We obtained a three-dimensional (3D) density reconstruction (Fig. 1e) at a resolution of 17 Å, which shows a bowl-shaped structure with dimensions of 80 × 48 × 40 Å. From this reconstruction, we were able to calculate the approximate molecular mass that fits into the density to be 62 kDa (Supplementary Fig. 3; see "Methods"). Therefore, the small AβO species, as visible on the micrographs, likely contains six dimAβ monomers (total MW of 60.2 kDa), which corresponds to 12 Aβ40 units. Dodecameric Aβ oligomers were observed before in AβO preparations from synthetic peptide or isolated from AD brain or mouse models and have been associated with neuronal dysfunction and memory impairment[55–58].

AβO formation occurred on the same time scale in the plate reader experiment as in microcentrifuge tubes (Fig. 1b, c, f). In contrast, extensive amyloid formation was observed in the plate reader experiment after ~10 h but was not detectable when AβOs were incubated in microcentrifuge tubes for several days, unless the microcentrifuge tube was agitated (Fig. 1b, c, f). This suggests that the movement of the microplate in the plate reader, caused by scanning of the wells during measurements every 3 min and 2 s of preceding orbital shaking, creates sufficient agitation to promote amyloid fibril nucleation. When the samples in the microplate were covered with a layer of mineral oil, AβO formation was unaffected but amyloid fibril formation was completely abrogated (Supplementary Fig. 4), in line with the essential role of the air–water interface in Aβ amyloid formation in vitro[59]. The strong effects of agitation[14] and air–water interface on Aβ amyloid fibril formation but not on AβO formation confirms again that their assembly mechanisms are different and is in line with the notion that AβO formation does not involve a nucleation step[11,60]. When AβOs, formed by incubation of dimAβ above the COC, were diluted to sub-COC concentrations, they persisted for >24 h, indicating high kinetic stability (Supplementary Fig. 5). We conclude that AβOs formed from dimAβ under quiescent conditions are kinetically stable, not replaced by amyloid fibrils for several days, and can be applied at sub-μM concentrations. DimAβ AβOs may therefore serve as a favorable AβO model.

To test whether dimAβ AβOs cause the same biological effects as reported for AβOs formed from Aβ40 or Aβ42, we investigated their binding to dendritic spines, their direct cytotoxicity, their capacity to induce Tau missorting, and their consequences for neuronal function. AβOs were formed from 20 μM dimAβ and added to primary mouse neurons (days in vitro 15 (DIV15)–22) to a final concentration of 0.5 μM (all dimAβ AβO concentrations given in dimAβ equivalents). One micromolar Aβ40 was used as monomeric control. DimAβ localized to neuronal dendrites both after 3 and 24 h of treatment, where it partially co-localized with dendritic protrusions positive for filamentous actin (stained by phalloidin), which mark synaptic spines (Fig. 2a). In contrast, Aβ40 monomers did not show substantial localization to dendrites (Fig. 2a). Direct cytotoxicity was assessed by analysis of the sizes and shapes of neuronal nuclei upon staining with NucBlue. The fractions of normal and dense nuclei did not change significantly after incubation with dimAβ AβOs (Fig. 2b, c), indicating the absence of direct cytotoxicity, in line with previous reports on AβOs[61].

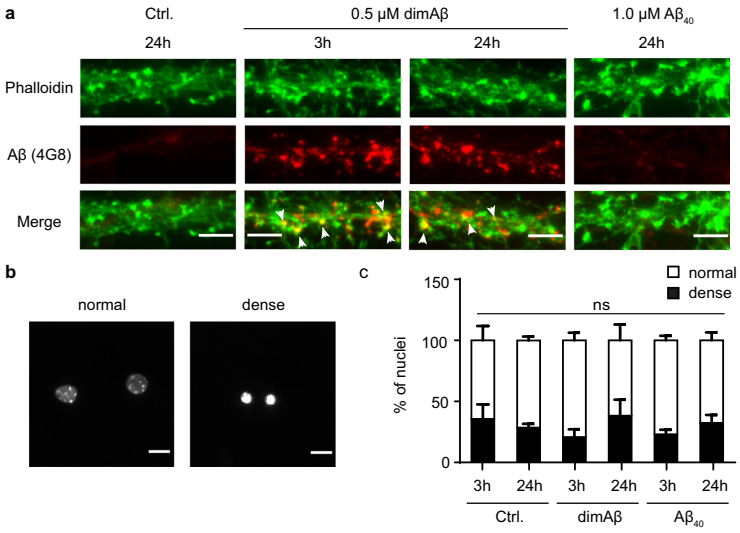

Fig. 2 DimAβ AβOs bind to dendrites and postsynaptic spines but have no direct cytotoxic effect on primary mouse neurons. Primary mouse neurons (DIV15–22) were treated with 0.5 µM dimAβ AβOs or 1 µM Aβ40 for 3 and 24 h. **a** DimAβ AβOs localized to neuronal dendrites both after 3 and 24 h of treatment, where they partially co-localized with phalloidin, a marker for synaptic spines. Arrows indicate co-localization of dimAβ with phalloidin. Scale bar, 5 µm. The experiment was independently repeated four times with similar results. **b** Nuclei of primary neurons were stained with NucBlue and analyzed with respect to shape and size. Representative images of normal and dense nuclei. Scale bar, 10 µm. **c** Quantification of normal and dense nuclei of primary neurons after vehicle control, Aβ40, or dimAβ AβO treatment revealed no direct cytotoxicity. $N = 3$; around 300 nuclei were analyzed for each condition. Error bars represent SEM. Statistical analysis was done by two-way ANOVA with Tukey's test for multiple comparisons and yielded no significant differences between the experimental groups.

Tau cellular distribution was analyzed with an anti-Tau (K9JA) antibody. DimAβ AβO-treated neurons showed strong enhancement of the fluorescence signal of Tau in the soma after 24 h of treatment (Fig. 3), indicating pathological somatodendritic Tau missorting as previously reported for AβOs[38,39]. In contrast, Aβ40 monomers did not induce Tau missorting in our experimental setting (Fig. 3). In previous studies, Tau missorting and spine loss were reversible within 12–24 h due to loss of AβO potency (transformation of AβOs over time to larger, non-toxic aggregates)[38,62]. Here we observe an increase of Tau missorting over time, which indicates remarkable kinetic stability and persistent ability of dimAβ AβOs to induce pathological Tau missorting.

Next, we investigated the consequences of AβO exposure for neuronal function. As readout, we measured spontaneous calcium oscillations in our neuronal cultures after dimAβ AβO treatment as an indicator for neuronal activity with live-cell imaging, using the fluorescent cell-permeable calcium indicator Fluo-4 as previously described[38]. A significant decrease of calcium oscillations was observed after 24 h but not after 3 h of treatment with dimAβ AβOs (Fig. 4). As calcium oscillations in our conditions depend on action potentials and neurotransmission, this indicates that dimAβ AβOs impair neuronal activity and function. With regard to dendritic spine binding, lack of direct cytotoxicity, potent induction of Tau missorting as well as decreased neuronal activity, dimAβ AβOs thus faithfully reproduce the observations previously made for AβOs formed from Aβ40 or Aβ42 or from 7:3 Aβ40:Aβ42 mixtures regarded as particularly toxic[38]. Of note, dimAβ AβOs effects appeared later (24 vs. 3 h) than for the previously studied oligomers, hinting toward their kinetic and structural stability in cell culture conditions.

Aβ42 as well as dimAβ accumulate within endo-lysosomal compartments. Next, we aimed to test the uptake of dimAβ AβOs in neuronal cells. First, SH-SY5Y neuroblastoma cells were subjected to a mixture of 0.1 µM HiLyte Fluor 647-labeled Aβ42 and 1 µM unlabeled Aβ42. After 24 h of incubation, Aβ42 accumulated within vesicular foci within the cytoplasm of the cells. Co-staining with a LysoTracker dye showed prominent colocalization suggesting the accumulation of Aβ42 within endo-lysosomal compartments (Fig. 5). This is in line with previous studies that showed Aβ42 accumulation in acidic vesicles of neuroblastoma cells and primary murine cortical neurons[41,44–46]. Hu et al. measured local Aβ42 concentrations >2.5 µM within endo-lysosomal compartments, which exceeds the extracellular concentration by approximately four orders of magnitude[44].

In a second attempt, SH-SY5Y cells were treated with 1.1 µM Abberior Star 520SXP-labeled dimAβ AβOs, formed from a mixture of 91% unlabeled and 9% fluorophore-labeled dimAβ (i.e., same final concentrations of unlabeled and fluorophore-labeled Aβ as in the Aβ42 experiment above). This experiment revealed a similar colocalization in acidic vesicles as for Aβ42 (Fig. 5). This confirms that both Aβ monomers and AβOs are readily taken up by neuron-like cells and accumulate in the endo-lysosomal system. Our results, however, do not reveal the assembly state of Aβ, and it is possible that the applied Aβ species undergo structural alterations upon cell entry and accumulation in endo-lysosomes, such as higher-order assembly as described below.

Endo-lysosomal pH promotes AβO assembly but delays amyloid fibril formation. Due to the accumulation of Aβ, endosomes/lysosomes might constitute the dominant site of the highly

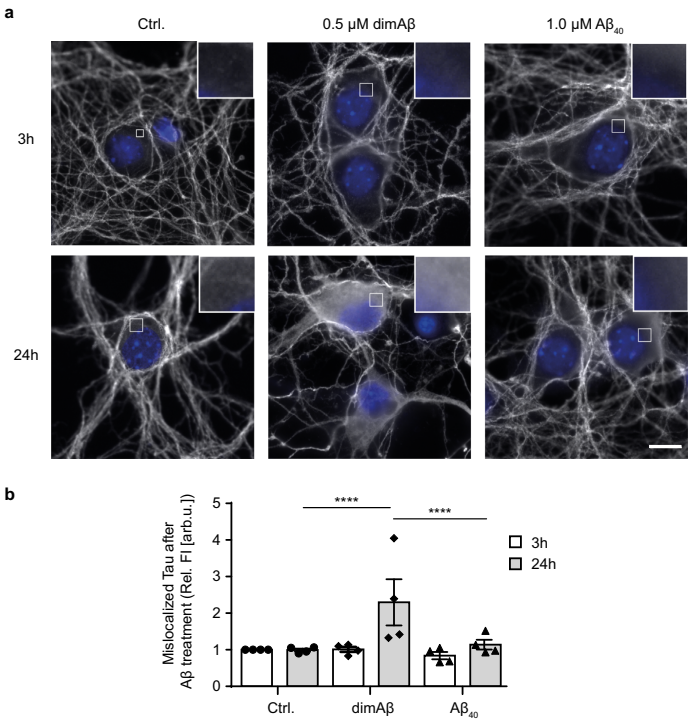

Fig. 3 DimAβ AβOs induce pathological somatodendritic missorting of Tau. Primary mouse neurons (DIV15–22) were treated with 0.5 µM dimAβ AβOs or 1 µM Aβ40 for 3 and 24 h. **a** Representative images of cell bodies of primary neurons after treatment with Aβ. Neurons were stained with anti-Tau (K9JA) antibody; nuclei were stained with NucBlue. DimAβ AβO-treated neurons show strong enrichment of fluorescence signal of Tau in the soma only after 24 h of treatment. Insets show magnification of white boxed areas in the somata. Scale bar, 10 µm. **b** Quantification of Tau enrichment in the soma of primary neurons. Fluorescence intensities of cell bodies were quantified and normalized to control-treated neurons after 3 h of treatment. $N = 4$, 30 cells were analyzed for each condition. Error bars represent SEM. Statistical analysis was done by two-way ANOVA with Tukey's test for multiple comparisons. Statistical significance: $^{****}p < 0.0001$.

concentration-dependent AβO formation. Apart from the increased Aβ concentration in endosomes/lysosomes, the low pH in late endosomes (~5.5) and lysosomes (~4.5) might promote AβO formation. We used dimAβ to simultaneously determine the specific effects of pH on AβO formation and on amyloid fibril formation. Lyophilized dimAβ was dissolved in 6 M buffered guanidinium chloride, followed by size-exclusion chromatography (SEC) into 1 mM NaOH, leading to a pH of 10.9, and added to the wells of a microplate. The basic pH conditions prohibit premature aggregation of Aβ[63]. The pH-dependent aggregation reaction was initiated in the microplate reader by injection of a 10× buffer yielding the desired final pH, allowing for monitoring of ThT fluorescence without any substantial delay. We determined the kinetics of dimAβ assembly between pH 4.8 and 7.6 in the concentration range 0.65–5.0 µM. At neutral pH, the initial kinetic phase reflecting AβO formation spanned several hours, but upon pH reduction, AβO formation was continuously accelerated and occurred within a few seconds at pH 4.8 (Fig. 6a–g). ThT fluorescence intensity decreased at acidic pH[64] but was still sufficiently sensitive to detect the signal of AβO formation at pH 4.8 and 0.65 µM dimAβ (Fig. 6g). For pH 7.4, we have previously shown that a global fit of an *n*th-order oligomerization reaction to the concentration-dependent assembly kinetics is in good agreement with the data and yields a reaction order of ~3.3 for dimAβ AβO formation[11]. Here we found that a reaction order of three applied to global fitting of the concentration-dependent data results in fits that reproduce the kinetic traces at all pH values (Fig. 6a–g). This indicates that the fundamental mechanism of AβO formation is not affected by pH reduction. A logarithmic plot of the obtained oligomerization rate constants against pH shows a linear trend with a slope of −1.56, i.e., the rate constant decreases 36-fold per pH unit within the investigated pH range (Fig. 6h). At pH 4.8, in between lysosomal and endosomal pH, AβO formation is 7900-fold faster than at interstitial pH (7.3).

In order to test whether the acceleration of AβO formation kinetics is accompanied by thermodynamic stabilization, we evaluated the effect of pH reduction on the COC of dimAβ. In the AβO formation assay at pH 7.4, the fluorescence intensity increase during the lag-free oligomerization phase scaled linearly with protein concentration at dimAβ concentrations above ~2 µM, whereas no lag-free oligomerization was detectable below ~0.5 µM, indicative of a COC of around 1 µM (Supplementary Fig. 6a, b). At pH 5.6, however, there is no indication of disappearance of the oligomerization phase down to a concentration of 0.4 µM dimAβ (Supplementary Fig. 6c, d). Due to the limited sensitivity of ThT at acidic pH[64], it is not possible to reliably monitor oligomerization at lower concentrations and to

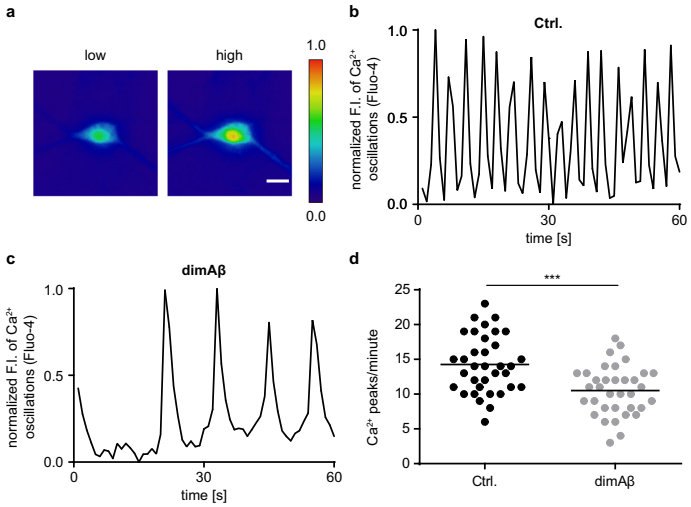

Fig. 4 DimAβ AβOs decrease spontaneous calcium oscillations of primary mouse neurons. Primary mouse neurons (DIV15–22) were treated with 0.5 µM dimAβ AβOs for 24 h. Cells were labeled with calcium-sensitive Fluo-4 dye and spontaneous calcium oscillations were recorded by time-lapse movies. **a** Representative ratiometric images of low and high calcium concentrations in the soma of a neuron. Scale bar, 20 µm. **b**, **c** Representative graphs of spontaneous Ca^{2+} oscillations in **b** vehicle control- and **c** dimAβ AβO-treated primary neurons. Fluorescence intensities were normalized to minimum values and plotted over time. **d** Quantification of spontaneous Ca^{2+} oscillations in primary neurons after vehicle control or dimAβ AβO treatment. Fluorescence intensities were normalized to minimum values and peaks per minute were counted for each sample. In total, 35 cells were analyzed; statistical analysis was done by two-tailed unpaired *t* test. Statistical significance: ***$p = 0.0001$.

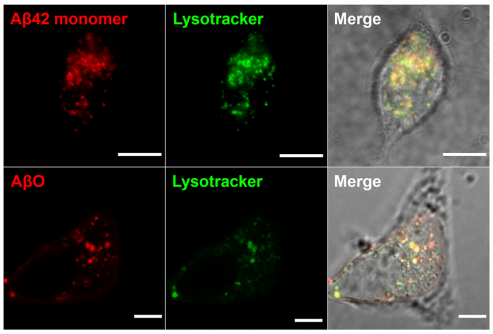

Fig. 5 Aβ42 and dimAβ AβOs accumulate in endosomes/lysosomes. SH-SY5Y cells were treated with Aβ42 monomers (top row) or dimAβ AβOs (bottom row) and co-localization with endo-lysosomal compartments was analyzed. 1.1 µM Aβ42 (containing 9% HiLyte 647-labeled Aβ42, top row) or 1.1 µM dimAβ AβOs (in monomer equivalents, formed from a dimAβ solution containing 9% AbberiorStar 520SXP-labeled dimAβ, bottom row) were added to the cells. After 24 h, the medium was exchanged with fresh medium supplemented with 50 nM Yellow HCK-123 LysoTracker dye. Scale bar, 5 µm. $N = 3$, at least three images were acquired for each treatment to ensure reproducibility.

determine the COC at this pH. Nevertheless, the COC at pH 5.6 is clearly lower than the COC at neutral pH, indicative of thermodynamic stabilization of AβOs at acidic pH.

AβOs formed at different pH values were imaged by AFM (Fig. 6i–o). From pH 7.6 to pH 6.8, AβOs were mainly spherical and curvilinear structures, the latter apparently resulting from bead-chain-like association of the spherical AβOs[6]. At pH 6.4, AβOs showed an increased tendency to form more compact structures, such as annular protofibrils and denser clusters. Below pH 6.0, AβOs associated into large clusters, in line with a previous description of Aβ40 aggregates at pH 5.8[53]. In AFM, these AβO clusters have average heights of ~100 nm, compared to heights of ~4 nm observed for AβOs formed between pH 6.0 and 7.2 (Fig. 6p). Thus, while the fundamental mechanism of AβO formation seems to be unaffected by pH reduction, there is an additional level of particle aggregation involved below pH 6.0.

The second kinetic phase in the ThT time course of dimAβ aggregation reports on amyloid fibril formation[11]. It is characterized by a lag time, which reflects the primary and secondary nucleation events involved in nucleated polymerization[14,52]. In contrast to the acceleration of AβO formation, the lag time of amyloid formation did not decrease with decreasing pH. On the contrary, the amyloid fibril formation phase could not be observed within 10 h experiments at pH values of 6.8 and below. This can be explained by the inhibition that the rapidly forming AβOs entail on amyloid formation: First, AβOs compete for the monomer growth substrate of amyloid fibril growth; second, AβOs actively inhibit amyloid fibril growth[11,65].

AβO assembly of Aβ42 is enabled under endo-lysosomal conditions. We investigated whether the promotion of AβO formation at endo-lysosomal pH is sufficient to also support AβO formation from Aβ42 at relevant endo-lysosomal Aβ concentrations, determined to be well above 2.5 µM[44]. At pH 7.2, Aβ42 in the concentration range 1.9–9 µM displayed sigmoidal assembly kinetics typical for amyloid fibril formation (Fig. 7a). The absence of a lag-free oligomerization phase is in agreement with the observation that the COC of Aβ42 in in vitro assay at neutral pH

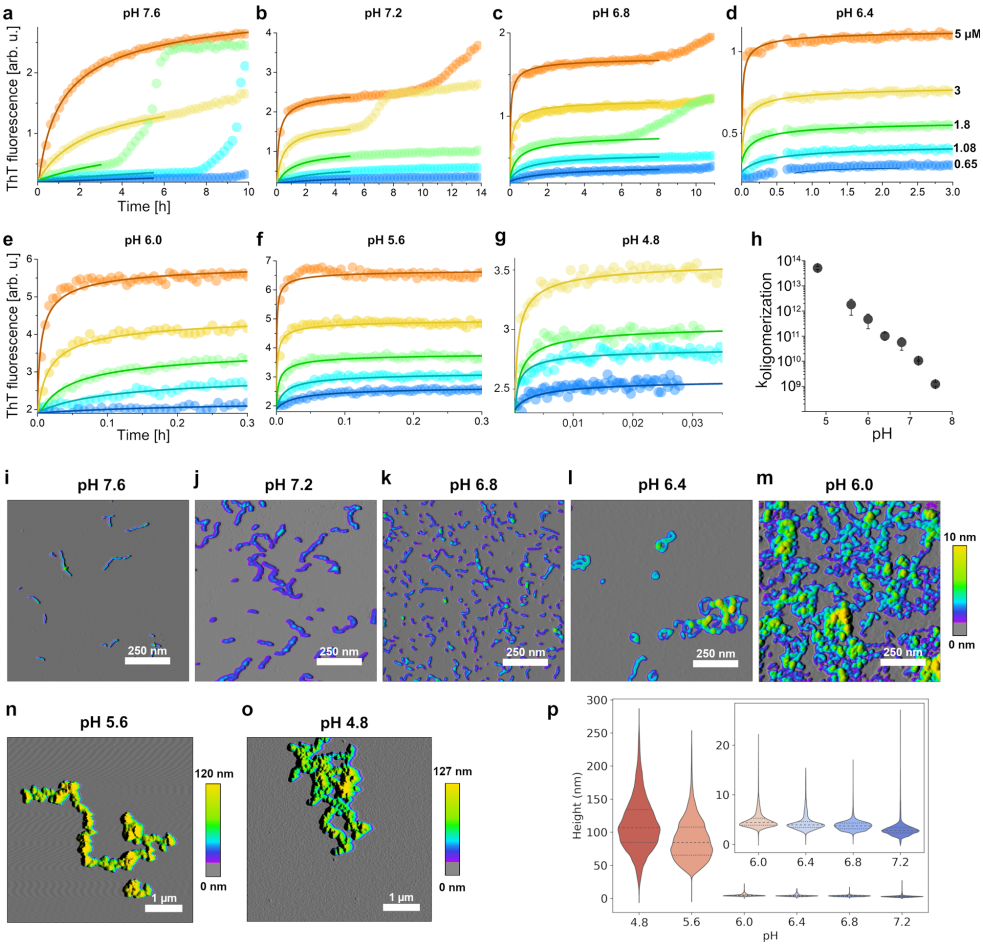

Fig. 6 pH dependence of dimAβ assembly kinetics. a–**g** DimAβ assembly at concentrations between 0.65 and 5 µM and at pH values between 4.8 and 7.6 monitored by ThT fluorescence. Solid lines represent global fits to the data using a one-step oligomerization model with a shared reaction order of 3 for all pH values and concentrations and an individual oligomerization rate constant per pH value. **h** Logarithmic plot of the obtained oligomerization rate constants vs. pH. The rate constants were obtained from global fits to *n* concentration dependence data sets obtained from *m* independently prepared assays, with *n*/*m* being 2/2 (pH 4.8), 6/4 (pH 5.6), 8/4 (pH 6.0), 5/4 (pH 6.4), 6/2 (pH 6.8), 6/2 (pH 7.2), and 6/2 (pH 7.6). One of the *n* repeats is shown in **a**–**g**. Replicates are given in Supplementary Fig. 8. Data points represent mean and standard deviation, except for pH 4.8, where the error bar indicates the higher and lower value of the $n = 2$ experiments. **i**–**o** AFM images of dimAβ AβO formed at different pH values. Note the dramatic change in the height scale bar upon pH decrease to <6.0 due to formation of large AβO clusters. Between 7 and 25 micrographs of at least 2 independent assays were recorded for each pH value to ensure reproducibility. **p** Particle height distributions determined from AFM images, displayed as violin plots. All pixels assigned to AβOs by the image analysis software in five micrographs per pH value were evaluated. Dashed lines represent medians; dotted lines represent interquartile ranges. Inset, zoom on the data for pH 6.0 to pH 7.2.

is >10 µM[65]. Consequently, the aggregation products under this condition are amyloid fibrils (Fig. 7c, f). In contrast, at pH 4.5 lag-free aggregation occurred at a concentration of ≥5.4 µM (Fig. 7b). The change from lag-containing to lag-free conditions at pH 4.5 was accompanied by a switch in aggregate morphology from amyloid fibril networks to large AβO clusters identical to those observed for dimAβ at endo-lysosomal pH (Fig. 7d, e, g, h). This indicates that under endo-lysosomal conditions the local Aβ concentration can exceed the COC of AβO formation, suggesting that endosomes/lysosomes may represent crucial sites of AβO formation in vivo.

Aβ aggregates can leak from endosomes/lysosomes into the cytosol and to other cell compartments or can be secreted and spread to other cells, potentially contributing to the propagation of Aβ pathology[27,28,44,45,51]. Upon transfer from endosomes/lysosomes to the cytosol or interstitial fluid, AβOs experience a shift from acidic to neutral pH. We tested the kinetic stability of AβOs formed at pH 4.5 after a shift to neutral pH by monitoring

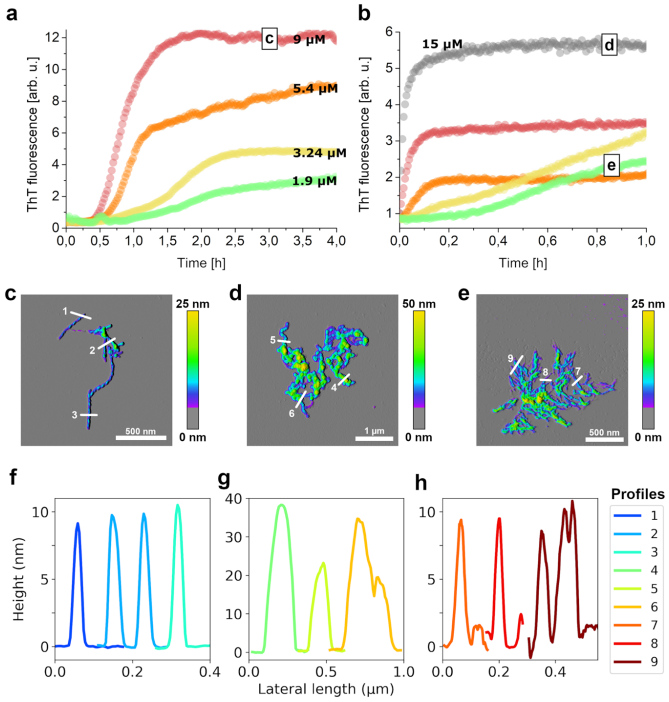

Fig. 7 Aβ42 rapidly forms AβOs at endo-lysosomal pH. a, **b** Aβ42 assembly at **a** pH 7.2 or **b** pH 4.5 at concentrations between 1.9 and 15 µM monitored by ThT fluorescence. Replicates are given in Supplementary Fig. 9. **c**–**e** AFM images of **c** amyloid fibrils formed by 9 µM Aβ42 at pH 7.2, **d** AβOs formed by 15 µM Aβ42 at pH 4.5, and **e** amyloid fibril networks formed by 1.9 µM Aβ42 at pH 4.5. At least three micrographs each of two independently prepared sample repeats were recorded to ensure reproducibility of the AFM data. **f**–**h** Height profiles of the sections indicated in **c**–**e**.

the ThT intensity and by imaging of the aggregate morphology by AFM. We applied Aβ42 at a concentration of 10 µM in this experiment, as Aβ42 does not form AβOs de novo at this concentration at neutral pH. Any AβOs observed after the pH shift can therefore safely be ascribed to the kinetic stability of AβOs pre-formed under acidic conditions. As before, a pH shift from basic pH to pH 4.5 was applied to initiate AβO formation. After AβO formation had reached a steady state, pH was adjusted to 7.2 by a further injection of a corresponding buffer stock. After the adjustment to neutral pH, there was an instantaneous increase in ThT fluorescence (Supplementary Fig. 7), which can be explained by the pH dependence of ThT fluorescence[64]. Thereafter, the ThT fluorescence did not exhibit any other larger changes that would be expected in the case of disassembly of AβOs or replacement of AβOs by an alternative type of aggregate. Apart from dense clusters like those observed for low pH AβOs, AFM images showed spherical and curvilinear structures typical for AβOs formed at neutral pH, indicating dissociation of the AβO clusters into their constituents (Fig. 8a). In fact, the AFM images suggest that smaller AβOs detach from fraying AβO clusters. The height of the cluster-released Aβ42 AβOs was 3.5–4.5 nm as measured by AFM in the dried state (Fig. 8b, c), identical to that of Aβ42 AβOs (Fig. 8d, e) and dimAβ AβOs (Fig. 6p) that were directly formed at neutral pH. Taken together, the ThT and AFM data demonstrate that AβOs formed at endo-lysosomal pH possess a high kinetic stability after shifting to neutral pH, which is, however, accompanied by dissociation of large AβO clusters into spherical and curvilinear AβOs.

Discussion

AβOs have been identified as the main neurotoxic Aβ species in AD. The characterization of the most critical disease-related AβOs has revealed that they are metastable oligomers >50 kDa in size that do not represent intermediates of amyloid fibril formation but are an alternative Aβ assembly type. However, the conditions required for AβO formation and the underlying mechanism have not been elucidated in detail. Here we show that AβO formation is highly pH dependent and is accelerated ~8000-fold upon a change in pH from neutral to endo-lysosomal pH. At the same time, the COC of AβO formation is reduced. This enables AβO formation at physiologically relevant Aβ concentrations, determined to be well above 2.5 µM in endo-lysosomal vesicles[44]. The strong acceleration of AβO formation at pH 4.5–5.5 suggests that the endosomal/lysosomal system might be a major site of AβO formation. AβOs may either form from Aβ monomers that have been newly generated by amyloid precursor protein (APP) processing or from endocytosed monomers (Fig. 9)[40–42,44,47,48]. APP processing in endo-lysosomal compartments by γ-secretase containing presenilin 2 generates a prominent pool of intracellular Aβ that is enriched in Aβ42 (ref. [48]). Esbjörner et al. applied fluorescence lifetime and

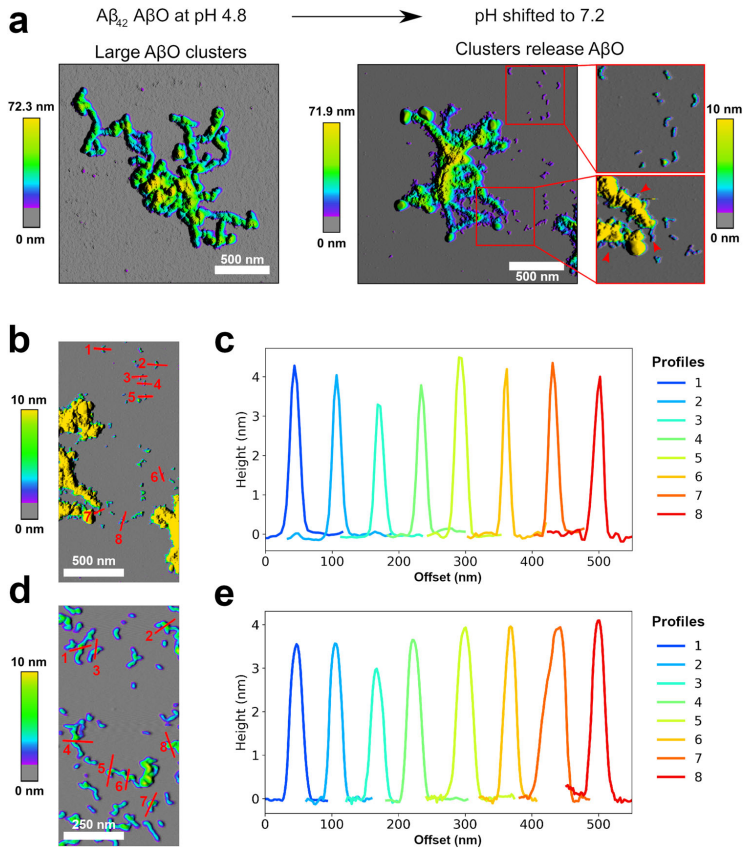

Fig. 8 Stability of AβOs formed by Aβ42 at endo-lysosomal pH after shifting to neutral pH. a AFM images of AβOs formed by 10 µM Aβ42 at pH 4.5 before (left) and after (right) shift to pH 7.2. Red arrowheads point to a few of the sites where AβOs seem to detach from AβO clusters. In all, 3–7 micrographs were recorded per condition to ensure reproducibility. **b**, **c** Height profiles of small AβOs after pH shift to neutral pH. Height profiles in **c** correspond to the sections in **b**. **d**, **e** Height profiles of AβOs formed by 110 µM Aβ42 at pH 7.2. Height profiles in **e** correspond to the sections in **d**.

super-resolution imaging to determine the kinetics of Aβ aggregation in live cells and found that aggregation occurred in endo-lysosomal compartments[41]. Importantly, they reported that Aβ42 aggregated without a lag time into compact, dense structures[41]. Both the absence of a lag time and the structural characterization are in line with the low pH AβO clusters described here, suggesting that AβO clusters indeed form in endo-lysosomal compartments and represent the dominant Aβ aggregate species in live cells. Subsequently, AβOs might cause lysosomal impairment, leak into the cytosol and cause intracellular damage, or might be secreted and spread to neighboring cells, where they could contribute to the propagation of pathology[40,42,44–46,48,49].

Enhanced aggregation at acidic pH is a known property of Aβ with established relevance for sample preparation[63]. Our results are in line with a study on the aggregation of Aβ40 (at a concentration of 230 µM) at pH 5.8 that reported the rapid formation of large clusters with (proto)fibrillar and globular substructures that were not able to seed, but rather inhibited, amyloid fibril formation[53]. Our analysis of the aggregation kinetics reveals that these low pH Aβ aggregates, often termed amorphous aggregates, form along the same pathway as neutral pH AβOs and therefore represent particle aggregates of AβOs. This is supported by the observation that low pH AβO clusters release spherical and curvilinear AβOs upon a shift to neutral pH (Fig. 8a). Nevertheless, there may be differences between atomic-level structures and between intermolecular interactions in AβOs formed at different pH, just as atomic-level structures and protofilament interfaces of amyloid fibril polymorphs can differ significantly.

The increasing clustering of AβOs upon pH reduction from neutral to pH 6 points to the high propensity of AβOs to associate. At neutral pH, self-association of spherical AβOs results in curvilinear assemblies. A decrease of pH leads to an increase in annular and compact assemblies and finally to large AβO clusters (Fig. 6). This propensity of AβOs to associate likely also contributes to their clustering with neuronal receptors[35,36] and to their accumulation around amyloid fibril plaques[66].

In contrast to AβO formation, amyloid fibril formation of dimAβ is slowed down at acidic pH. This pH dependence is not an inherent property of Aβ amyloid fibril formation: in the absence of AβOs, Aβ42 amyloid fibril formation occurs rapidly at

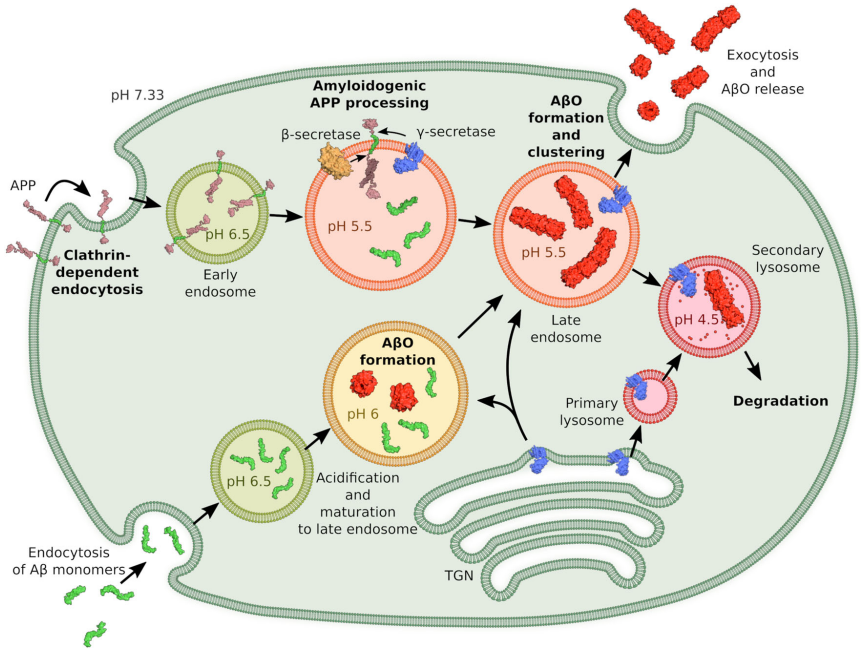

Fig. 9 Scheme of intracellular APP processing, Aβ uptake, and AβO formation. This is an extension of previous schemes of APP processing and Aβ uptake[48,76,77], now including potential formation of AβOs especially in endo-lysosomal compartments. Using a conservative estimate of the endo-lysosomal Aβ concentration of 2.5 μM[44] and assuming an endosome volume of 0.3 μm^3, there are on average 450 Aβ molecules in an endosome. Protein structure images were prepared using pdb entries 1OWT, 1IYT, 1RW6, 3DXC, 4UIS, and 1SGZ. TGN trans-Golgi network.

pH 4.5 (Fig. 7b, 1.9 μM trace). Delayed amyloid fibril formation upon pH reduction is only observed in combination with accelerated AβO formation and can be explained by the two inhibitory activities of AβOs on amyloid fibril formation: AβOs compete with amyloid fibrils for monomers (Fig. 1a) and furthermore inhibit amyloid fibril growth actively[11,65].

DimAβ AβOs show dendritic spine binding, lack direct cytotoxicity, potently induce Tau missorting, and decrease neuronal activity, suggesting that they constitute a suitable AβO model construct to study the pathomechanism of AD. Previous AβO preparations showed a loss of potency to induce Tau missorting within 12 h due to transformation to non-toxic larger Aβ aggregates[38,62]. In contrast, dimAβ AβOs led to extensive and persistent Tau missorting 24 h after application. The sustained activity of dimAβ AβOs is likely a consequence of the kinetic stabilization of the AβO state achieved by the dimer linkage. DimAβ might therefore be an advantageous model for eliciting Tau missorting and downstream consequences, as it represents a model of chronic stress corresponding to the human disease rather than acute insult.

Methods

Preparation of dimAβ. DimAβ was produced recombinantly[11]. Expression of dimAβ was achieved by co-expression of ZAβ3, a binding protein that shields aggregation-prone sequence segments of Aβ[67]. The gene encoding dimAβ included an N-terminal methionine, followed by a Aβ40 unit, a $(G_4S)_4$ linker, and a second Aβ40 unit. DimAβ and $(His)_6$-tagged ZAβ3 were co-expressed from a pACYCDuet-1 vector that contained the genes in the following order: T7promoter-1–dimAβ–T7promoter-2–$(His)_6$ZAβ3–T7 terminator. BL21(DE3) *E. coli* cells (Novagen) were transformed with the expression vector and grown for ~16 h at 37 °C on LB agar plates containing 34 μg/ml chloramphenicol. Single colonies were picked and grown for ~16 h in 50 ml M9 medium, containing 2×YT medium and 34 μg/ml chloramphenicol. In all, 40 ml of the pre-culture was transferred to 2 l of M9-Celtone medium in a 5 l baffled Erlenmeyer flask. The culture was grown at 37 °C with shaking and induced at OD_{600} ~ 0.8 by the addition of IPTG to a final concentration of 1 mM. After further growth for 4 h, the cells were harvested and frozen at −20 °C.

For purification, cell pellets were resuspended in 50 mM Na-phosphate, 0.3 M NaCl, 20 mM imidazole, pH 8, containing EDTA-free protease inhibitor (Roche Applied Sciences), and lysed by a cell disrupter (Constant Systems). The cell debris was removed by centrifugation in a Beckman J2-21 centrifuge mounting a JA20.1 rotor at 18,000 RPM, 4 °C for 40 min. For capture of the dimAβ:ZAβ3 complex by immobilized metal ion affinity chromatography (IMAC), the supernatant was loaded on a HisTrap FF column (GE Healthcare). DimAβ was separated from the resin-bound ZAβ3 and eluted with 8 M urea and 20 mM Na-phosphate, pH 7. For further purification, including removal of residual ZAβ3, reverse-phase high-performance liquid chromatography (RP-HPLC) was performed. For this purpose, the IMAC eluate was concentrated in a Vivaspin 20 centrifugal concentrator (Sartorius), followed by addition of 5 mM TCEP to reduce the disulfide bond of ZAβ3, and loading onto a semi-preparative Zorbax 300SB-C8 RP-HPLC column (9.4 mm × 250 mm, Agilent) connected to an Agilent 1260 Infinity system with UV detection at 214 nm. Monomeric dimAβ was eluted in a gradient from 30% (v/v) to 36% acetonitrile in water and 0.1% (v/v) trifluoroacetic acid at 80 °C. DimAβ-containing fractions were pooled, lyophilized, dissolved in hexafluoroisopropanol (HFIP), aliquoted in 1 mg portions, lyophilized again, and stored at −20 °C.

For aggregation kinetic experiments, the lyophilized protein was reconstituted in 6 M guanidinium chloride and 50 mM sodium-phosphate buffer, pH 7.4, and incubated at room temperature (RT) for 30 min. Subsequently, SEC was performed using a Superdex 75 increase column (GE Healthcare) equilibrated with 1 mM NaOH. The concentration of the monomeric dimAβ in the alkaline eluate was measured via tyrosine fluorescence using a pH-adjusted extinction coefficient of 2685 M^{-1} cm^{-1}. Samples were always kept on ice until further needed.

ThT aggregation kinetics. ThT, NaN_3, NaCl, protein, and 1 mM NaOH were given into the wells of a 96-well low-binding plate (Greiner) such that if filled up to 100 μl, concentrations of 1 μM ThT, 0.02% NaN_3, 150 mM NaCl, and the desired final protein concentration were reached. The outermost wells of the plate were left

blank due to the risk of aberrant aggregation behavior. The plate was put in a BMG ClarioStar platereader fitted with two injectors and tempered at 37 °C. One syringe of the injector was equilibrated with 1 ml 10× buffer concentrate. The reaction was started using the injector of the platereader by dispensing 10 µl of the concentrate at highest available speed into each of the wells. This adjusted the pH value in situ and initiated oligomerization. Data points were collected in evenly spaced intervals depending on the velocity of the reaction using the BMG Reader Control software (version 5.40).

For shifting the pH in situ twice, both syringes were equilibrated with 10× buffer concentrate; the first one resulting in a final buffer concentration of 20 mM and pH 4.5 and the second one resulting in a final buffer concentration of 50 mM and pH 7.2. The first syringe was used to inject 10 µl to initiate oligomerization, whereas the second one was used to inject 11 µl to achieve the shift to neutral pH at a time point where the oligomerization reaction had reached its plateau.

For analysis of the kinetics of AβO formation, the initial phase of the ThT kinetics was fit to one-step oligomerization $nM \rightarrow M_n$ (ref. [11]). The AβO mass concentration, $M_{AβO}$, evolves in time according to the following expression

$$M_{AβO}(t) = M_0 - [M_0^{1-n} + (n-1)nkt]^{1/(1-n)} \quad (1)$$

with M_0 the total protein concentration, k the oligomerization rate constant, and n the oligomer size or reaction order. Global fits to the pH- and concentration-dependent AβO formation data were performed using the Origin 9.0 software with a reaction order of $n = 3$ shared between all data sets, and the oligomerization rate constant k as a pH-dependent parameter, which was shared within the concentration-dependency data sets at a given pH. The proportionality constant relating $M(t)$ to ThT fluorescence intensity was treated as a fit parameter with an individual value for every sample.

Atomic force microscopy. In all, 10 µl of the dimAβ samples were taken directly from the plate after the ThT assays at a concentration of 5 µM and applied onto freshly cleaved muscovite mica. They were left to dry, washed with 500 µl ddH_2O, and dried with a stream of N_2 gas. For imaging dimAβ at pH 4.8, the aforementioned method did not work, likely due to sticking of the sample to the well. Instead, all reaction components apart from the buffer concentrate were premixed and loaded into a micropipette tip. By adding the reaction components to a vial containing the buffer concentrate and thorough mixing, the reaction was started, before pulling the solution back into the tip. Immediately afterwards, the micropipette was relocated into a 37 °C incubation cabinet, where a drop was pushed out to the point where it still stuck to the tip. After 45 s, the drop was pushed onto the freshly cleaved muscovite mica and preparation commenced as with the other pH values.

For the Aβ42 samples, 5 µl of the respective concentrations were taken, applied onto freshly cleaved muscovite mica, and left to dry for 15 min before carefully washing with 200 µl ddH_2O and drying under a stream of N_2 gas.

Imaging was performed in intermittent contact mode (AC mode) in a JPK Nano Wizard 3 atomic force microscope (JPK, Berlin) using a silicon cantilever with silicon tip (OMCL-AC160TS-R3, Olympus) with a typical tip radius of 9 ± 2 nm, a force constant of 26 N/m, and resonance frequency around 250 kHz. The images were processed using the JPK DP Data Processing Software (version spm-5.0.84). For the presented height profiles, a polynomial fit was subtracted from each scan line first independently and then using limited data range. False-color height images were overlaid onto the amplitude profile.

Particle height distributions were extracted from AFM images. Therefore, the Morphological Active Contours without Edges (MorphACWE) function of python's scikit-image module was used to distinguish and separate AβOs from background (see Supplementary Fig. 10 for examples of AFM image segmentation). Histographical height profiles of AβOs at different pH were determined as per pixel heights of the MorphACWE-isolated areas.

Cryo-EM. For cryo-EM imaging, the AβO sample was plunge-frozen on glow-discharged Quantifoil 1.2/1.3 grids. In total, 1308 micrographs were recorded as focal pairs at high defocus (6 µm) and low defocus (using a range of −0.5 to −2 µm) on a Tecnai Arctica (200 kV) using a Falcon III direct electron detector, yielding a pixel size of 0.935 Å. Particle selection was performed automatically using crYOLO[68]. In total, 32,211 particles were selected on the high defocus micrographs. The contrast transfer function of the micrographs was determined using CTFFIND4[69]. Further image processing was performed using the software package RELION 3.0.5[70]. Two-dimensional and 3D classification was conducted on the high-defocus images to clean the data set. A box size of 128 pix, which corresponds to 119.7 Å, and a radial mask with a diameter of 100 Å were used.

The high-defocus micrographs were aligned to the low-defocus micrographs. The relative shifts obtained from this alignment were applied to all particles (that were picked from the high-defocus micrographs) and then the particles were extracted from the low-defocus micrographs with the shifted particle coordinates, while keeping the Euler angles from the high-defocus 3D refinements. A 3D reconstruction calculated from the high-defocus images was low-pass filtered to 60 Å and was used as an initial model for further low-defocus 3D refinements. For further processing steps, only micrographs that contain a signal beyond a resolution of 5 Å were used. The final resolution of 17 Å was assessed by Fourier shell correlation.

In order to obtain an estimate for the molecular mass within the reconstructed density, 110 pseudo-atomic models with varying number of pseudo-atoms (molecular masses between 10 and 120 kDa) were generated from the density map using the program VISDEM[71], which is part of the software package DireX[72]. In VISDEM, atoms are randomly placed into a density region with density above a provided threshold. The density threshold was set to yield a volume such that the mass density is fixed at 0.714 ml/g (average mass density observed in proteins). The pseudo-atomic model has a composition of 62.2% C atoms, 20.6% O atoms, and 17.2% N atoms, which corresponds to the average composition observed in proteins. Afterwards, a density map was computed from each of the 110 pseudo-atomic models. The VISDEM method was used to sharpen these pseudo-atomic model maps as well as the EM reconstruction. The sharpening was performed with a resolution cutoff of 17 Å and the mass of the corresponding pseudo-atomic model. Finally, the cross-correlation between the sharpened EM reconstruction and the sharpened pseudo-atomic model map was computed and plotted for each tested mass. The highest cross-correlation was found for the pseudo-atomic model map that contains a molecular mass of 62 kDa. One dimAβ monomer (101 amino acids) has a molecular mass of 10.0 kDa. Thus, the reconstructed density likely holds six dimAβ monomers. The final 3D reconstruction of the oligomer was sharpened by VISDEM using a mass of 62 kDa and a resolution cutoff of 17 Å.

Preparation of dimAβ AβOs and Aβ40 monomers for treatment of primary neurons. Aβ preparations were performed under sterile conditions. DimAβ lyophilisate was resuspended in 50 mM NaOH until completely dissolved. Next, phosphate-buffered saline (PBS) and 50 mM HCl were added and immediately mixed, obtaining a final concentration of 20 µM dimAβ and 40 µM Aβ40. To induce AβO formation, dimAβ was incubated at 37 °C for 16 h. Aβ40 controls were prepared in the same manner without subsequent incubation. Primary neurons (DIV15–22) were treated with either 0.5 µM dimAβ AβO or 1 µM Aβ40 monomers diluted in conditioned neuronal maintenance media for 3 and 24 h under normal growth conditions (see below). In addition, control cells were treated with a vehicle control (PBS containing 50 mM NaOH and 50 mM HCl). Afterwards, cells were fixed and stained as described below.

Primary neuron culture. Primary neurons were isolated and cultured as described before[73] with slight modifications: In brief, the brains of FVB/N mouse embryos were dissected at embryonic day 13.5. Brainstem and meninges were removed and whole cortex was digested with 1× Trypsin (Panbiotech). Neurons were diluted in pre-warmed (37 °C) neuronal plating medium (Neurobasal media (Thermofisher Scientific), 1% fetal bovine serum (FBS; Biochrom AG), 1× antibiotic/antimycotic solution (Thermofisher Scientific), 1× NS21 (Panbiotech)) and seeded onto poly-D-lysine (Merck) coated coverslips. Neurons were cultivated in a humidified incubator at 37 °C, 5% CO_2. Four days after plating, media was doubled with neuronal maintenance media (Neurobasal media (Thermofisher Scientific), 1× antibiotic/antimycotic solution (Thermofisher Scientific), 1× NS21 (Panbiotech)) and cells were treated with 0.5 µg/ml Cytosine β-D-arabinofuranoside (AraC; Sigma-Aldrich). The isolation of primary neurons was reviewed and approved (§4 TschG) by the Animal Welfare Officer of University of Cologne and the Landesamt für Natur-, Umwelt- und Verbraucherschutz (LANUV), Germany.

Somatodendritic missorting of Tau. To analyze Tau somatodendritic localization, neurons were fixed with 3.7% formaldehyde/4% sucrose in PBS (both Sigma-Aldrich) for 30 min at RT using gentle agitation after treatment with Aβ or vehicle control for the indicated time points. Afterwards, cells were permeabilized and blocked for 5–10 min in 5% bovine serum albumin/0.2% TX-100 in PBS (both Carl Roth), washed with PBS, and stained with a polyclonal rabbit anti-Tau (K9JA, Dako A0024; dilution: 1:1000) antibody overnight at 4 °C. The next day, coverslips were washed again with PBS, incubated with NucBlue (Thermofisher Scientific) for 15 min, and subsequently stained with a secondary antibody coupled to an AlexaFluor dye (Thermofisher Scientific) for 1 h at RT. Coverslips were mounted onto glass slides using Aqua-Poly/Mount (Polysciences) and dried overnight at RT (for further details on immunofluorescence staining procedure, see ref. [73]). Images of neuronal cell bodies were taken with a wide-field fluorescence microscope (Axioscope 5, Zeiss) and the ZenBlue Pro imaging software (V2.5, Zeiss). Fluorescence intensities of cell bodies were quantified using the ImageJ software[74,75]. Fluorescence intensity values were normalized to vehicle-treated control cells after 3 h of treatment. All experiments were performed 4 times; 30 cells were analyzed for each condition. Statistical analysis was done by two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons using GraphPad Prism v6 (GraphPad Software).

Cytotoxic effect of dimAβ. To evaluate AβO toxicity, cells were fixed and stained with NucBlue (Thermofisher Scientific) after dimAβ AβO treatment. Shape and density of nuclei were analyzed and counted: cells were considered dead, when nuclei appeared condensed and smaller, compared to viable cell nuclei. All experiments were conducted for 3 times; around 300 nuclei were analyzed for each condition. Statistical analysis was done by two-way ANOVA with Tukey's test for multiple comparisons using GraphPad Prism v6 (GraphPad Software).

Aβ targeting to postsynaptic spines and imaging of spontaneous calcium oscillations. To analyze Aβ binding to synapses, neurons were fixed and stained for F-actin with phalloidin as a marker of synaptic spines (Thermofisher Scientific) and a monoclonal mouse anti-Aβ (clone 4G8, Merck, #MAB1561; dilution: 1:300) antibody. The experiment was repeated independently for four times and colocalization of AβO with synapses was observed for all replicates.

To monitor spontaneous Ca^{2+} oscillations, primary neurons were labeled with 2 µM Fluo-4 (Thermofisher Scientific) and 0.02% Pluronic F127 (Merck) for 20 min after 24 h of dimAβ treatment. Time-lapse movies of different fields were recorded for 1 min each (frame rate: 1 s) using a Leica DMi8 microscope (Leica) and the Leica LAS X imaging software (v3.7.3). Fluorescence intensity changes of cell bodies were quantified over time with ImageJ[74,75] and corrected for background signal. Fluorescence intensities were normalized to minimum values and peaks per minute were counted for each sample. In total, 35 cells were analyzed; statistical analysis was done by two-tailed unpaired *t* test.

Preparation of fluorescently labeled Aβ for cell culture experiments. For preparation of AbberiorStar 520SXP-labeled Cys0-dimAβ, a mutant of dimAβ with an N-terminal cysteine residue was expressed as described above. For fluorophore labeling, TCEP-reduced Cys0-dimAβ lyophilisate was incubated in 200 mM HEPES pH 7.0 with a twofold molar excess of maleimide-conjugated AbberiorStar 520SXP fluorophore, which was dissolved in dimethylformamide. After 2 h of incubation, the labeled dimAβ was purified using reverse-phase HPLC. Samples were lyophilized, redissolved in HFIP, and aliquots were prepared. These aliquots were lyophilized and stored at RT for later use. Abberior STAR 520SXP-labeled AβOs were prepared from a 1:10 molar ratio of Abberior STAR 520SXP-labeled dimAβ and unlabeled dimAβ, in order to avoid that the fluorophore alters AβO properties. In all, 10 µl of 1:10 mixture of Abberior STAR 520SXP-labeled dimAβ and unlabeled dimAβ was prepared in 50 mM NaOH. Quickly, 490 µl phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin–streptomycin was added, and the pH was readjusted by adding 10 µl 50 mM HCl. The final dimAβ concentration was 10 µM. The sample was quiescently incubated at 37 °C in the dark for 24 h. AβO formation was confirmed using AFM.

For Aβ42 cell culture experiments, Aβ42-HiLyte Fluor 647 (Anaspec) was dissolved in HFIP and lyophilized into smaller aliquots (30 µg). For cell culture experiments, aliquots were first dissolved in 3 µl 50 mM NaOH. In all, 544 µl phenol red-free DMEM supplemented with 100 U/ml penicillin–streptomycin was added, and the pH was recalibrated by the addition of 3 µl 50 mM HCl. To avoid exposure of the Aβ peptide to local low pH environments, the HCl was pipetted into the lid of the tube, closed, and quickly vortexed. This procedure yields a 10 µM mostly monomeric stock solution of Aβ42-HiLyte Fluor 647 suitable for cell culture experiments.

Neuroblastoma cell culture. SH-SY5Y cells were grown to 80% confluency in DMEM with phenol red, 10% FBS, and 100 U/ml penicillin–streptomycin in T75 flasks. Experiments were performed in Ibidi collagen IV-coated µ-Slide VI 0.4. A total of 7500 cells (250,000 cells/ml) were seeded into each channel of the slide. Cells adhered to the surface of the channels within an hour of incubation at 37 °C, 5% CO_2, in a humidified chamber. Subsequently, the feeding reservoirs of the channels were filled with further medium. Each day, the medium in the reservoirs was replaced with fresh medium until the cell density was satisfactory for coincubation experiments.

Coincubation experiments and imaging. For coincubation and imaging experiments, phenol red was removed by flushing the channels three times with phenol red-free DMEM supplemented with 100 U/ml penicillin–streptomycin. Subsequently, channels were filled with medium containing corresponding Aβ species. Cells were incubated for 24 h. Channels were flushed with fresh medium and supplemented with 50 nM Yellow HCK-123 LysoTracker. Imaging was performed either on a Leica Infinity TIRF microscope or on a confocal microscope using the Leica LAS AF software. Confocal measurements were performed using a TCS SP8 STED 3× (Leica Microsystems) equipped with an HC PL APO CS2 ×100 objective (NA 1.4) at a scan speed of 600 Hz and a line accumulation of 6. A 488 nm of a pulsed white light laser was chosen as excitation for Yellow HCK-123 LysoTracker and AbberiorSTAR520XPS. The emitted fluorescent signal was detected by counting-mode hybrid detectors in the spectral range of 500–531 nm for Yellow HCK-123 LysoTracker and 650–765 nm for AbberiorStar520SXP. Additionally, a time-gating of 0.1 ns was used to avoid laser reflection.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The cryo-EM density map of dimAβ AβOs has been deposited in the Electron Microscopy Data Bank under accession code EMD-11327. The authors declare that all the data necessary to interpret, verify, and extend the research of the article are available within the article (and Supplementary Information files). All data are available from the corresponding authors on reasonable request. Source data are provided with this paper.

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Author contributions

M.P.S., F.H., S.B., G.F.S., H.Z., and W.H. designed the experiments. M.P.S., F.H., S.B., M. Z., S.H., G.F.S., H.Z., and W.H. performed the experiments and analyzed the data. M.P.S., F.H., S.B., M.Z., G.F.S., H.Z., and W.H. wrote the manuscript. All authors commented on the manuscript.

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Competing interests

The authors declare no competing interests.

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Endo-lysosomal Aβ concentration and pH trigger formation of Aβ oligomers that potently induce Tau missorting

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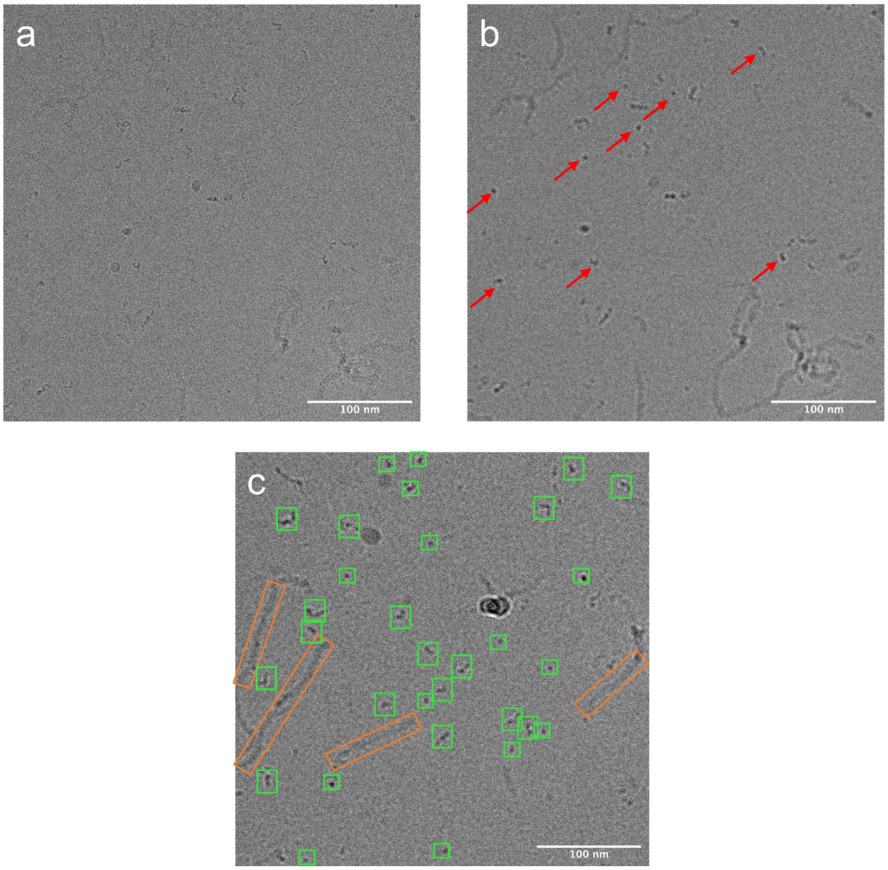
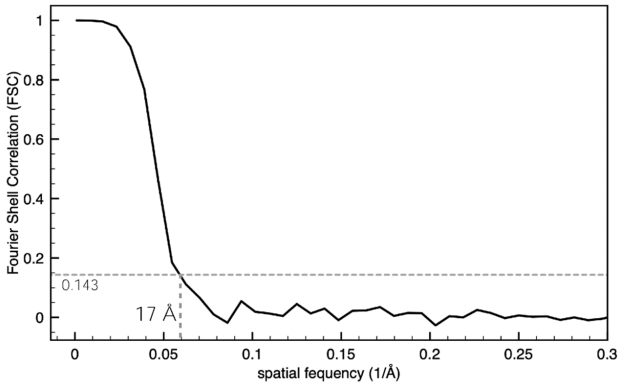
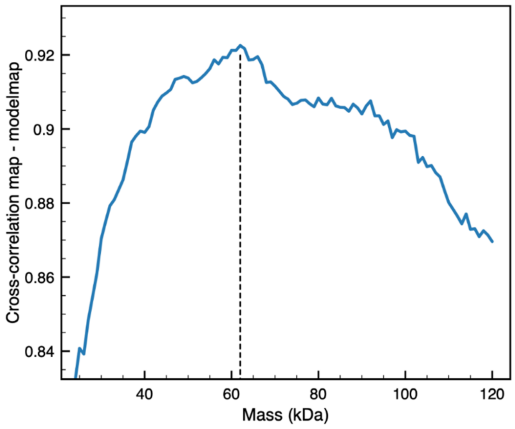
Supplementary Figures



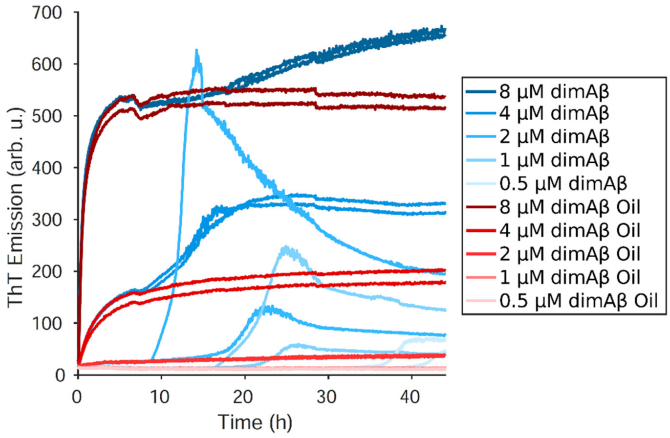
Supplementary Fig. 1 Representative examples from a total of 1308 cryo-EM micrographs collected of dimAβ AβOs. Micrographs were recorded at a defocus of **a** -1.6 µm or **b** -6 µm, respectively. Small AβO particles, indicated by red arrows, were selected on the high defocus micrographs for density reconstruction. **c** Thirty micrographs were analyzed to estimate the relative abundance of the small AβOs (green boxes) and AβO protofibrils (orange boxes). All clearly discernible objects were counted. Small AβOs accounted for 72±12% of all particles. Considering the particles' dimensions, we estimated that small AβOs contain 2-3% of all Aβ molecules within AβOs.



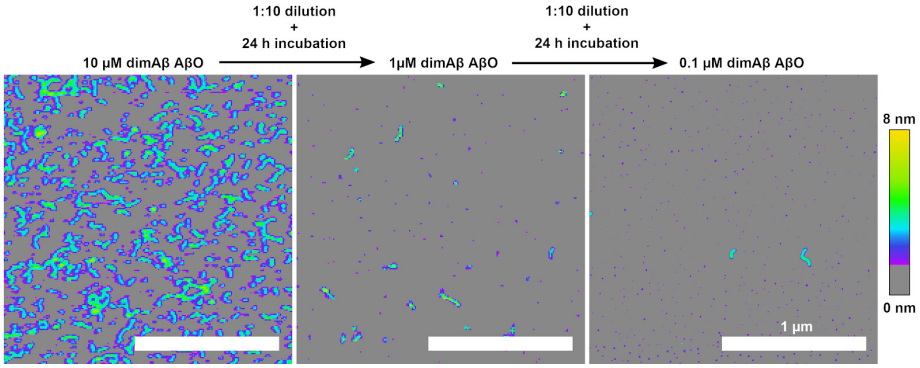
Supplementary Fig. 2 Fourier shell correlation (FSC) for the 3D reconstruction of the smallest dimAβ AβOs observed on the cryo-EM micrographs yields a resolution estimate of 17 Å.



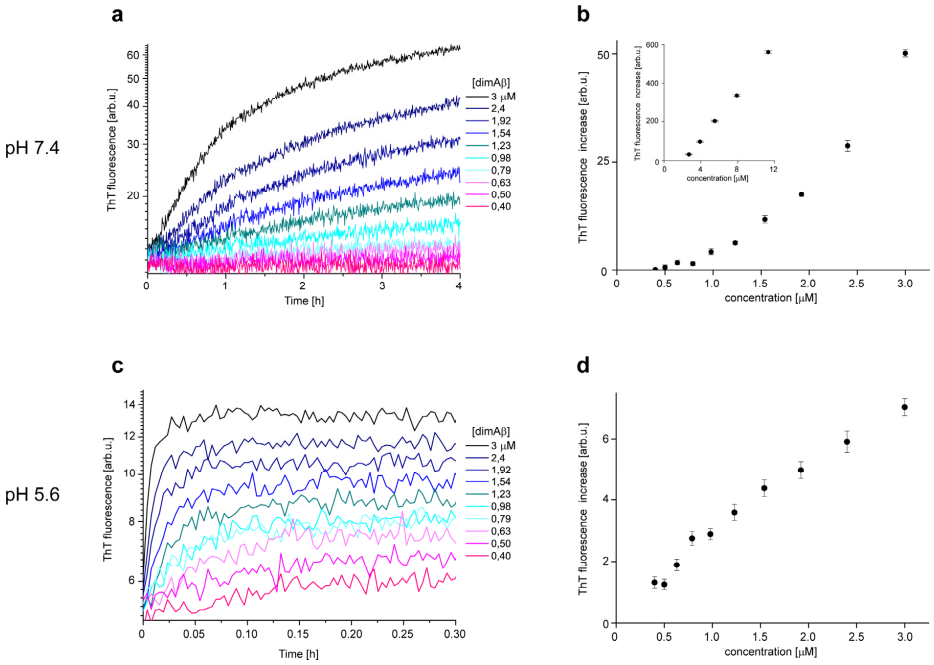
Supplementary Fig. 3 Density cross-correlation computed for each of the 110 pseudo-atomic model maps with the EM reconstruction after sharpening with VISDEM using the corresponding mass of the pseudo-atomic model. The highest correlation (0.923) is obtained for a mass of 62 kDa.



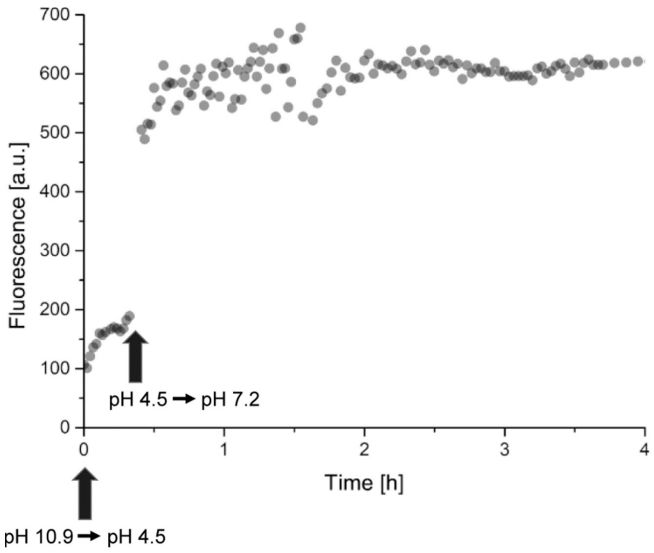
Supplementary Fig. 4 The air-water interface is crucial for fibril nucleation *in vitro*. Time courses of dimAβ assembly at pH 7.4 monitored by ThT fluorescence in a platereader. Half of the samples were covered by layering 10 µl of mineral oil on top of the aqueous solution. AβO formation was not impaired by mineral oil. Fibril nucleation, on the other hand, was retarded and not detectable during the whole timespan of the experiment.



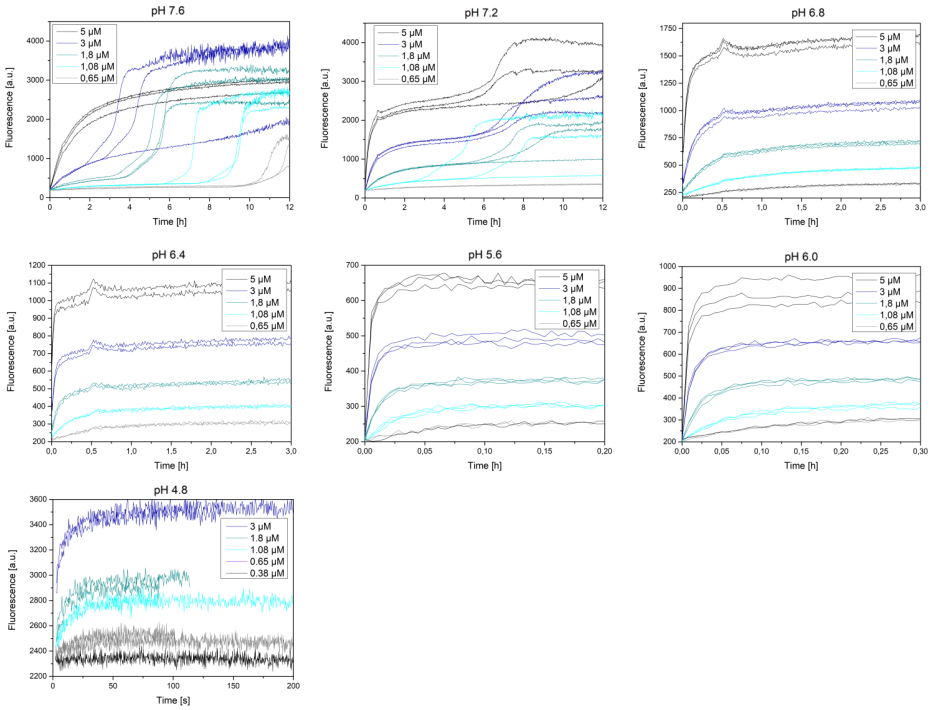
Supplementary Fig. 5 Long-term stability of diluted AβO. 10 µM dimAβ were quiescently incubated at 37°C, pH 7.4, for 72 h (left). Subsequently, the solution was diluted ten-fold to a dimAβ concentration of 1 µM and further quiescently incubated for 24 h at 37°C (middle). This solution was then further diluted ten-fold to a dimAβ concentration of 0.1 µM, which is far below the COC, and further incubated quiescently for 24 h at 37°C (right). Scalebar, 1 µm. *N*=1, between two and five micrographs were recorded for each condition.



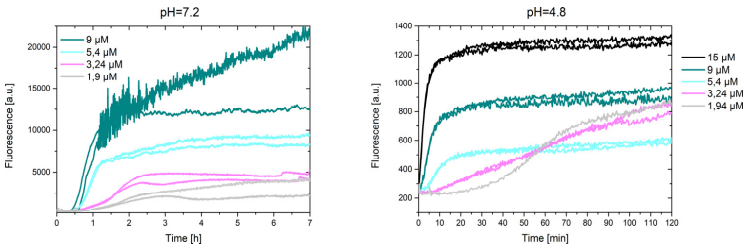
Supplementary Fig. 6 The critical concentration of AβO formation is reduced at acidic pH. Analysis of the AβO formation phase of dimAβ assembly at pH 7.4 (**a**, **b**) and pH 5.6 (**c**, **d**). **a**, **c** ThT fluorescence time traces at the indicated dimAβ concentrations. **b**, **d** The increase in ThT fluorescence intensity during the first 4 h (**b**) or 0.3 h (**d**) plotted against the dimAβ concentration. The inset in **b** shows data of a separate experiment spanning a range of higher concentrations. The data values in **b** and **d** are from single kinetic runs per concentration, with error bars representing the standard deviation of the final ten fluorescence readings in **a** and **c**. At pH 7.4, the intensity increase during the lag-free oligomerization phase scales linearly with protein concentration at dimAβ concentrations above ~2 µM (**b**, inset), whereas no lag-free oligomerization is detectable below ~0.5 µM, indicative of a COC of around 1 µM (**a**, **b**). At pH 5.6, however, there is no indication of disappearance of the oligomerization phase down to a concentration of 0.4 µM dimAβ (**c**, **d**). Due to the limited sensitivity of ThT at acidic pH it is not possible to reliably monitor oligomerization at lower concentrations and to determine the COC at this pH. Nevertheless, the COC at pH 5.6 is clearly lower than the COC at neutral pH, indicative of thermodynamic stabilization of AβOs at acidic pH.



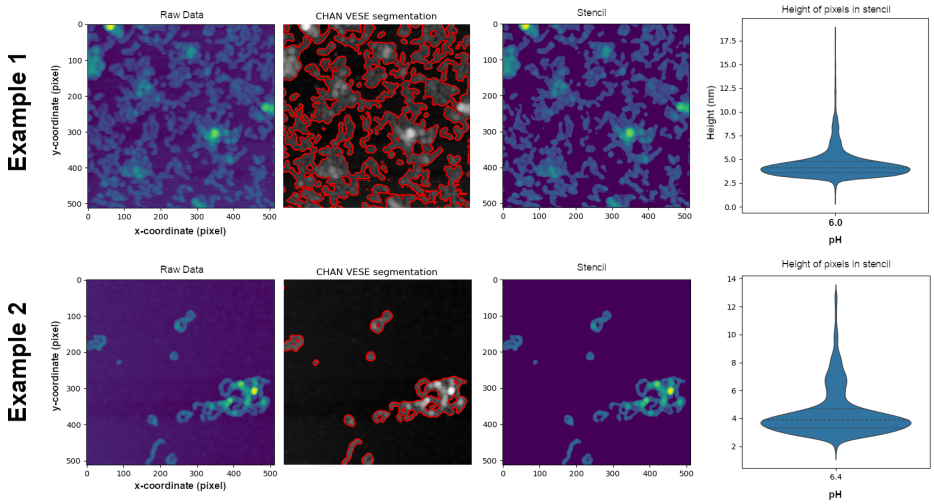
Supplementary Fig. 7 Stability of AβOs formed from Aβ42 at endo-lysosomal pH after shifting to neutral pH. ThT time course of AβO formation, initiated by pH adjustment from 10.9 to 4.5. Upon pH adjustment from pH 4.5 to pH 7.2 an immediate increase in fluorescence intensity is observed due to the pH sensitivity of ThT fluorescence. Apart from that, no other larger signal changes that would be expected in the case of disassembly of AβOs or replacement of AβOs by an alternative type of aggregate were observed.



Supplementary Fig. 8 Replicate time traces from the dimAβ assembly experiment of Fig. 6.



Supplementary Fig. 9 Replicate time traces from the Aβ42 assembly experiment of Fig. 7.



Supplementary Fig. 10 Examples of AFM image segmentation for height anaylsis. The Morphological Active Contours without Edges (MorphACWE) function 'morphological_chan_vese' of python's scikit-image module was used to distinguish and separate AβOs from background (typical settings were: iterations = 35; smooth = 1; lambda1 = 0.9; lambda2 = 0.89 to 0.895). Histographical height profiles of AβOs at different pH were determined as per pixel heights of the MorphACWE-isolated areas.

2.2. Schützmann & Hoyer (2025) Off-pathway oligomers of α-synuclein and Aβ inhibit secondary nucleation of α-synuclein amyloid fibrils, *Journal of Molecular Biology* 437, Issue 10

Contribution (First author): Planning and realization of all experiments, preparation of the figures, writing of the initial draft, revisions and reviewing of the manuscript.

Research Article

Off-pathway oligomers of α-synuclein and Aβ inhibit secondary nucleation of α-synuclein amyloid fibrils

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Abstract

α-Synuclein (αSyn) is a key culprit in the pathogenesis of synucleinopathies such as Parkinson's Disease (PD), in which it forms not only insoluble aggregates called amyloid fibrils but also smaller, likely more detrimental species termed oligomers. This property is shared with other amyloidogenic proteins such as the Alzheimer's Disease-associated amyloid-β (Aβ). We previously found an intriguing interplay between off-pathway Aβ oligomers and Aβ fibrils, in which the oligomers interfere with fibril formation via inhibition of secondary nucleation by blocking secondary nucleation sites on the fibril surface. Here, using ThT aggregation kinetics and atomic force microscopy (AFM), we tested if the same interplay applies to αSyn fibrils. Both homotypic (i.e. αSyn) and heterotypic (i.e. Aβ) off-pathway oligomers inhibited αSyn aggregation in kinetic assays of secondary nucleation. Initially soluble, kinetically trapped Aβ oligomers co-precipitated with αSyn(1–108) fibrils. The resulting co-assemblies were imaged as clusters of curvilinear oligomers by AFM. The results indicate that off-pathway oligomers have a general tendency to bind amyloid fibril surfaces, also in the absence of sequence homology between fibril and oligomer. The interplay between off-pathway oligomers and amyloid fibrils adds another level of complexity to the homo- and hetero-assembly processes of amyloidogenic proteins.

Introduction

Alpha-synuclein (αSyn), a 140 amino acid long protein[1] that makes up 0.5–1% of the total cytosolic protein in the brain,[2] represents a promising focal point for investigating Parkinson's Disease (PD) and other neurodegenerative diseases due to its involvement in both sporadic and familial forms.[3,4] It is implicated in multiple detrimental processes to afflicted cells, from oxidative stress stemming from mitochondrial dysfunction to membrane disruption.[5–7] *In vitro*, αSyn presents as an intrinsically disordered protein, but it can assume highly-ordered β-sheet arrangements upon assembly into amyloid fibrils.[8–10]

Whereas monomeric αSyn is hardly implicated in toxicity, the focus has shifted from amyloid fibrils to smaller species that are now more often implied to have adverse effects.[11,12] This shift is also true for proteins featured in other amyloidoses, such as amyloid-β (Aβ), which is normally associated with Alzheimer's disease (AD).[13] Distinct amyloidogenic proteins are regarded as a significant factor in the pathogenesis of distinct neurodegenerative diseases, such as αSyn in the range of synucleinopathies. However, they often show clinical and

pathological overlap with other amyloidogenic proteins or other neurodegenerative diseases, respectively.[14,15] For example, >50% of AD patients exhibit not only Aβ plaques and Tau tangles but also αSyn pathology, and Aβ plaques are frequently encountered in synucleinopathy cases.[16,17] The pathological overlap might be a result of a direct interaction between αSyn and Aβ, which may result in cross-seeding of fibril formation.[18–23]

Analysis of cross-interactions of different amyloidogenic proteins is hampered by the diversity of aggregate species.[24,25] Oligomeric species can either appear as on-pathway intermediates of amyloid fibril formation or as metastable off-pathway structures that have to disassemble into monomers before continuing into fibrils (see Fig. 1A).[26–28] Especially off-pathway oligomers exhibit detrimental activities in line with early pathogenic events in neurodegeneration. For example, αSyn oligomers (αSynOs) impair synaptic function, lead to increased intracellular Ca^{2+} levels, and disturb mitochondria as well as cellular proteostasis.[29] Similarly, Aβ oligomers (AβOs) are synaptotoxic and lead to cognitive decline in mouse and non–human primate models.[13,30–33] Consequently, they present an interesting target for therapeutic approaches, as in the case of the monoclonal anti-Aβ antibody lecanemab, which was developed to exhibit particularly high affinity for protofibrillar off-pathway oligomers.[34]

We have recently characterized a model for protofibrillar Aβ off-pathway oligomers formed from the dimeric Aβ variant dimAβ.[27,35] In dimAβ, two Aβ40 units are linked in one polypeptide chain through a flexible glycine–serine-rich linker. DimAβ preferentially forms AβO (termed here dimAβO) due to the increased local Aβ concentration, allowing to study effects of AβO with minimized disturbance by Aβ monomers or fibrils. Importantly, dimAβO recapitulate the biophysical and functional properties of native AβO.[35] Application of dimAβO revealed an intriguing interplay between Aβ off-pathway oligomers and Aβ fibrils, beyond the mere competition for Aβ monomers: Off-pathway oligomers bind to the fibril surfaces, which impedes secondary nucleation, i.e. the auto-catalytic formation of new fibril nuclei on the surface of existing fibrils (Fig. 1).[36]

Secondary nucleation has been identified as a critical step in amyloid formation of Aβ, αSyn and many other amyloidogenic proteins, and is considered to contribute to the spreading of amyloid pathology.[37,38] The identified interplay of off-pathway oligomers and amyloid fibrils entails that the two species mutually influence their time evolution, which affects the extent of their toxic activities.[36] For example, amyloid fibril plaques may serve as a reservoir for neurotoxic Aβ oligomers.[39]

Here, we investigated if the oligomer-fibril interplay previously observed for Aβ and lysozyme also applies to αSyn. In particular, we wanted to test if sequence identity of the proteins that constitute the off-pathway oligomers or amyloid fibrils, respectively, is required for this interplay. Therefore, we tested the effects of both

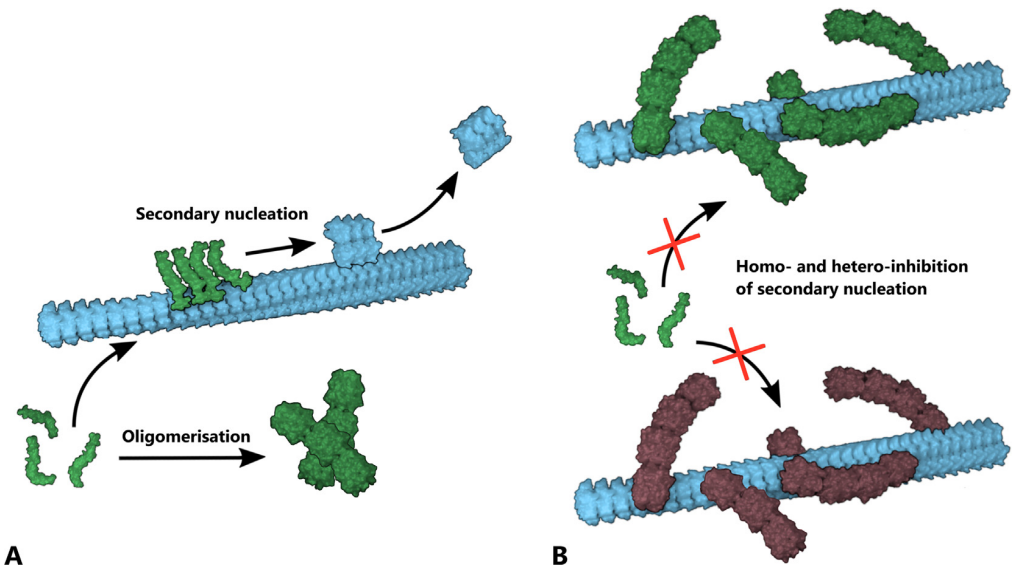

Fig. 1. Suggested mechanism of the inhibition of secondary nucleation by off-pathway oligomers. (A) Scheme of fibril formation by secondary nucleation versus off-pathway oligomer formation. (B) Off-pathway oligomers impede secondary nucleation by binding to fibril surfaces and masking secondary nucleation sites.

homo-oligomers (i.e. αSynO) and hetero-oligomers (dimAβO) on αSyn fibril formation.

Results

Both homo- and hetero-amyloid oligomers inhibit αSyn secondary nucleation

Secondary nucleation drives aggregation of full-length αSyn in particular under slightly acidic conditions, with C-terminal truncation shifting the pH window of dominant secondary nucleation into the neutral pH range.[40,41] To investigate the effect of homo- and hetero-oligomers on secondary nucleation of full-length αSyn we therefore performed aggregation assays at a pH of 5.0. We have previously shown that at this pH, in the presence of low concentrations of pre-formed fibril seeds, the monomer concentration dependence of the aggregation kinetics is in agreement with a driving role of secondary nucleation.[42] In the aggregation assays an αSyn monomer concentration of 70 μM and quiescent conditions were chosen in order to ensure the critical role of secondary nucleation. Test reactions in the absence of pre-formed fibril seeds or in the presence of the nucleation-specific inhibitor AS69fus[42] confirmed the critical role of secondary nucleation under the chosen experimental conditions. (Fig. 2).

αSynO (homo-oligomers) were prepared according to the protocol for off-pathway αSyn oligomer preparation by dialysis against water, lyophilisation, subsequent resuspension and separation by SEC.[43–45] In atomic force microscopy (AFM) αSynO are imaged as spherical structures that persist under the experimental conditions of the secondary nucleation assay (Fig. 3, top row).

As hetero-oligomers, Aβ oligomers were chosen due to the possibly disease-relevant interaction of Aβ and αSyn.[14–23] Oligomers formed by the synthetic Aβ dimer dimAβ, which consists of two Aβ40 units linked in one polypeptide chain, were chosen as oligomer model. DimAβ forms kinetically stable oligomers that reliably mimick off-pathway Aβ oligomers.[27,35] Application of dimAβ enables facilitated control over the multimerisation state, as it assembles into oligomers at low micromolar concentrations and does not convert into fibrils in the absence of agitation.[27,35] At neutral pH, dimAβO are spherical and curvilinear species[27,35] (Fig. 3, bottom row). When the pH is decreased to the pH of the secondary nucleation assay of full-length αSyn, these species cluster into large aggregates (Fig. 3, middle row). We have previously shown that these dense aggregates are indeed assemblies of small AβO.[35]

We first tested if αSynO inhibit the proliferation of αSyn fibrils. 70 μM αSyn monomer was incubated in the presence of 100 nM preformed αSyn fibril seeds and increasing concentrations of αSynOs. The aggregation time courses showed a prolongation of the lag-time in dependence of the αSynO concentration (Fig. 4A), indicating that αSynOs inhibit secondary nucleation of αSynO amyloid fibrils. This is in agreement with Yang et al., who observed that αSyn off-pathway oligomers inhibited seeded αSyn aggregation.[46] Similarly, Lorenzen et al. showed that certain αSyn off-pathway oligomers inhibit αSyn amyloid formation.[44]

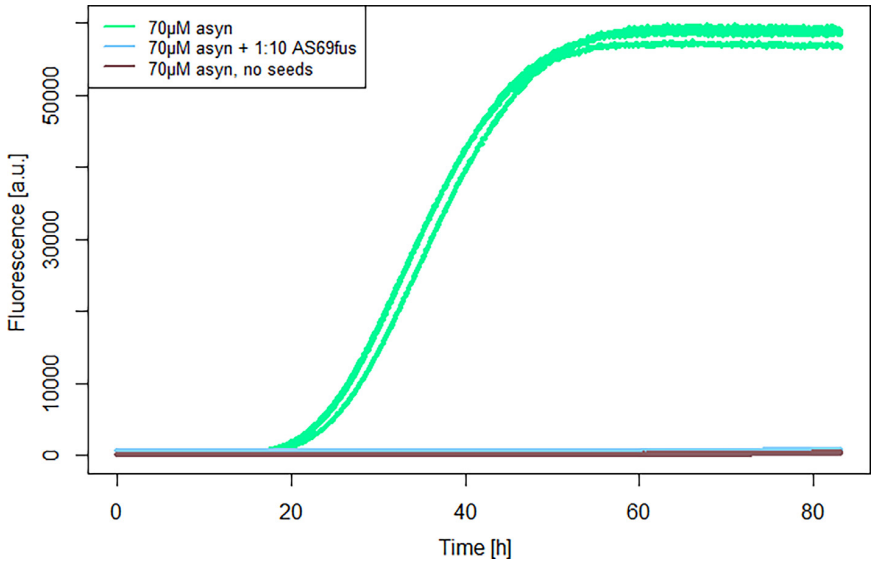

Fig. 2. Quiescent αSyn secondary nucleation assay at pH 5.0. 70 μM αSyn was incubated in the presence of 100 nM preformed αSyn fibril seeds. No aggregation was detectable in the absence of seeds or in the presence of the inhibitor AS69fus, which inhibits secondary nucleation under these conditions.[42]

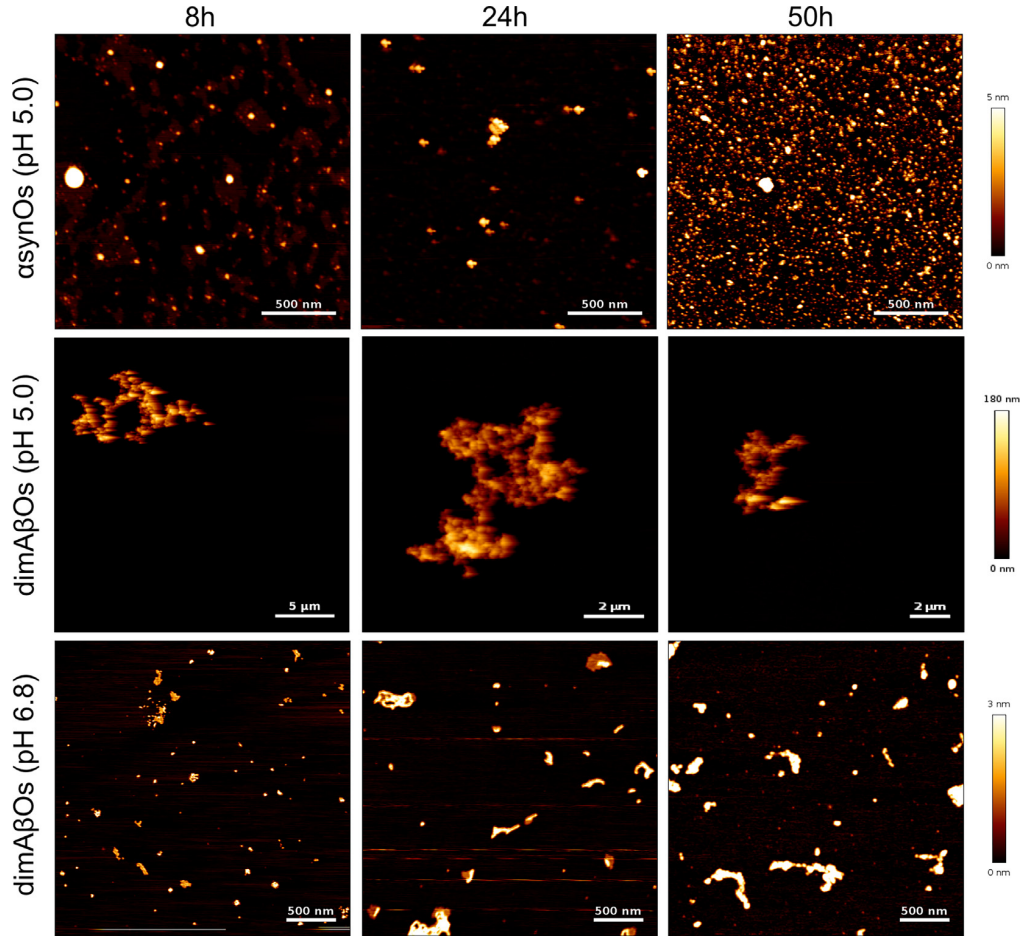

Fig. 3. AFM of oligomers applied in this study. Oligomer morphology and stability over time were investigated under the solution conditions of the secondary nucleation assays. Top row, αSynOs at pH 5.0; middle row, dimAβOs at pH 5.0; bottom row, dimAβOs at pH 6.8. Note that the clusters of dimAβO at pH 5.0 are of far greater height than the other oligomer species.

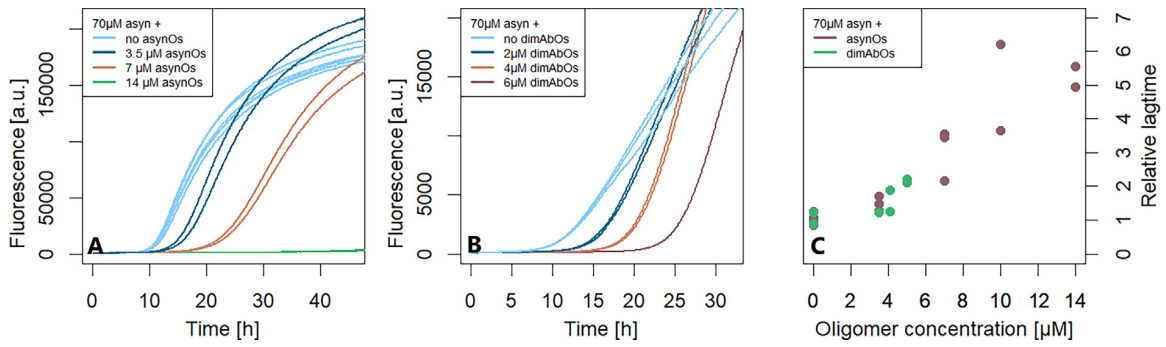

Fig. 4. Off-pathway oligomers increase the lag-time of αSyn secondary nucleation kinetics. Kinetics of secondary nucleation assays in presence of αSynO (A) or dimAβO (B). Both assays were done under quiescent conditions at pH 5, incorporating 70 µM of monomer and 100 nM of seeding fibrils, which were bath-sonicated before. (C) Dependence of lag-times on the concentration of off-pathway oligomers. The lag-times were extracted from the linear part of all ThT curves recorded at pH 5 and then normalized on the lag-time of the samples containing no added oligomeric species.

Having confirmed the inhibitory effect of off-pathway oligomers of one protein on amyloid fibrils of the same protein (i.e., homo-inhibition) we went on to investigate if cross-protein inhibition (i.e., hetero-inhibition) also occurs. When different concentrations of dimAβOs were added to 70 μM αSyn monomer seeded with 100 nM αSyn fibrils, a prolongation of the lag-time to an extent similar to the one caused by αSynOs was observed (Fig. 4B and C). This indicates that sequence identity is not required for amyloid oligomers to inhibit amyloid fibril formation. The data furthermore suggest that the clustering of dimAβOs at pH 5.0 does not abrogate their potential to inhibit secondary nucleation of fibrils.

DimAβOs inhibit αSyn1–108 secondary nucleation by binding to fibrils

Previously, we reported a direct interaction between dimAβO and Aβ fibrils.[36] This direct interaction could explain the inhibitory effect of Aβ oligomers on Aβ fibril formation, as the oligomers may block the sites of secondary nucleation on the amyloid fibril surface (Fig. 1B). Here, we aimed to investigate if the same mechanism could apply to the cross-inhibition of αSyn secondary nucleation by dimAβO. However, detection of the oligomer-fibril interaction was dependent on the separation of fibril-bound and free oligomers by way of faster sedimentation of the fibrils in comparison to free oligomers. This previously allowed us to pull down oligomers with fibrils and then image the complex by atomic force microscopy (AFM).[36] Here, however, at the acidic pH used in the αSyn secondary nucleation experiment dimAβOs assemble into larger clusters and therefore display similar sedimentation behavior to αSyn fibrils, prohibiting separation (Fig. 3, middle row).[35] Therefore, the assay was adapted by employing instead of wild-type αSyn a variant lacking the C-terminus (αSyn1–108). This variant shows secondary nucleation at a higher pH,[41] at which dimAβOs do not clump and hence do not sediment by themselves.

To confirm that secondary nucleation is indeed active and inhibited by αSynO under these conditions, we first repeated the ThT aggregation assays with αSyn1–108 at pH 6.8, both under quiescent (Fig. 5A) and shaking (Fig. 5B) conditions, both with and without addition of dimAβOs. The data displayed an oligomer concentration-dependent increase of lag-time (Fig. 5A,B) similar to the experiments with wt αSyn done at pH 5.0, confirming that αSyn1–108 is a suitable model for investigating effects on secondary nucleation at an increased pH of 6.8.

Since monitoring of secondary nucleation in the kinetic assay strictly requires fibril elongation to occur in addition to secondary nucleation, we next ruled out that dimAβ oligomers affected fibril elongation. A setup was chosen that enforces specifically this process: By utilizing quiescent conditions and relatively high concentration of 10% seeding fibrils (in monomer equivalents), which were sonicated to increase the number of fibril ends, the elongation process was dominant, as shown in Fig. 5C. The addition of 2 and 5 μM dimAβOs, which respectively constitutes 8 and 20% of the added monomer (comparable to the addition of 5 and 14 μM dimAβOs in the secondary nucleation assays) did not show an effect on the initial increase in ThT fluorescence, which reflects the elongation rate (Fig. 5C). The data support that dimAβOs inhibit αSyn1–108 fibril formation by interfering with secondary nucleation but not with fibril elongation.

After showing that αSyn1–108 is a suitable αSyn variant to observe the inhibitory effect of dimAβOs on αSyn secondary nucleation at neutral pH, a potential direct interaction of dimAβOs with αSyn1–108 fibrils was investigated by AFM. Preformed αSyn1–108 fibrils were coincubated with dimAβOs at pH 6.8, followed by centrifugation and subsequent imaging of both supernatant and

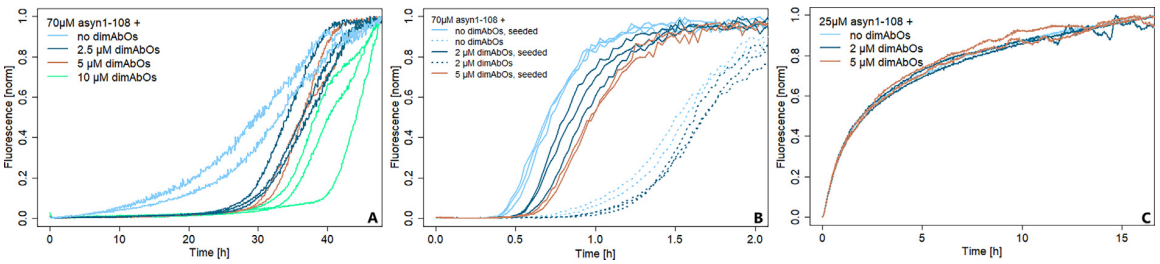

Fig. 5. DimAβ oligomers interfere with secondary nucleation but not with elongation of αSyn1–108 fibrils. (A,B) Kinetics of secondary nucleation assays of αSyn1–108 with 100 nM seeds in the presence of varying concentrations of dimAβO under quiescent (A) or under shaking (B) conditions. (C) Kinetics of fibril elongation of 25 μM αSyn1–108 with 2.5 μM seeds under quiescent conditions in the presence of varying concentrations of dimAβO. All assays were performed at pH 6.8.

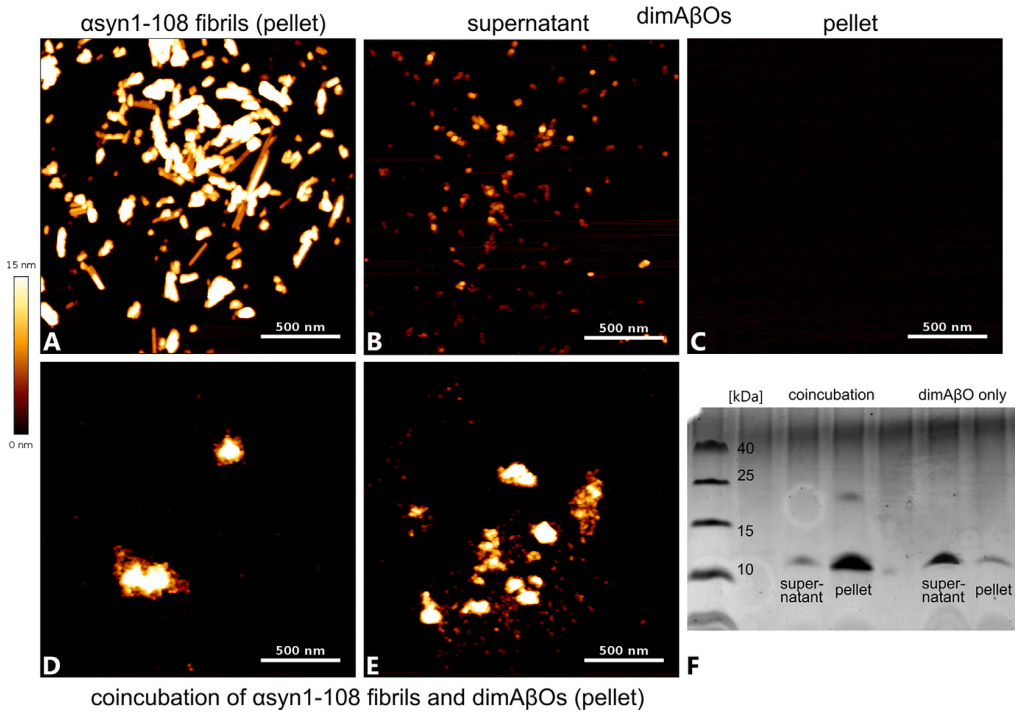

Fig. 6. DimAβOs bind to αSyn1–108 fibrils. Fibrillar αSyn1–108 aggregates and dimAβO were prepared separately and analyzed either individually (A-C,F) or after conincubation (D-F). Upon centrifugation, pellets and supernatants were analyzed by AFM (A-E) and SDS–PAGE (F). DimAβ is visible as a band at 11 kDa in SDS–PAGE. In SDS–PAGE of the coincubation sample, αSyn1–108 fibrils did not enter the gel; αSyn1–108 is therefore not detectable.

resuspended pellet. Both αSyn1–108 fibrils and dimAβOs were imaged separately as controls.

As reported before, αSyn1–108 formed short fibrils with a tendency to cluster into bundles that were found in the pellet (Fig. 6A).[47] In contrast, dimAβOs remained in the supernatant as curvilinear assemblies (Fig. 6B-C). The height of dimAβOs was lower than that of αSyn1–108 fibrils and fibril bundles. Upon coincubation, spherical and curvilinear assemblies of the height of dimAβOs were found in the pellet, suggesting that dimAβOs were co-precipitated by αSyn1–108 aggregates (Fig. 6-D-E). This finding is supported by SDS–PAGE analysis of the partitioning of dimAβ into the supernatant and the pellet fractions. Upon coincubation with αSyn1–108 aggregates, the largest part of dimAβ shifted into the pellet fraction (Fig. 6F). In the AFM images of coincubation samples, the spherical and curvilinear assemblies were associated with aggregates of larger height, which likely correspond to αSyn1–108 fibril clusters (Fig. 6D-E). In contrast to αSyn1–108 aggregates imaged in absence of dimAβOs, individual fibrils were not detectable in the coincubation samples. This suggests that dimAβOs promote further clustering of αSyn1–108 fibrils into dense aggregates. Taken together, AFM and SDS–PAGE of the conincubation samples provides evidence of a direct interaction of αSyn1–108 fibrils with dimAβOs, as observed before in the homotypic case of Aβ fibrils and dimAβOs.

Discussion

Here, we find that off-pathway αSyn oligomers interfere with secondary nucleation of αSyn fibrils. This interference with secondary nucleation can explain the previously observed inhibitory effect of αSynOs on fibril formation.[46,44] In addition, by combining a well-established αSyn secondary nucleation assay with the well-characterized Aβ off-pathway oligomer model dimAβ, we were able to observe that heterotypic (i.e., Aβ) oligomers inhibit αSyn secondary nucleation to a similar extent as homotypic (i.e., αSyn) oligomers. With regard to the mechanism underlying inhibition of secondary nucleation, pull-down and AFM experiments show that dimAβO binds to fibril surfaces (Fig. 6). The resulting obstruction of secondary nucleation sites on fibril surfaces can well explain the inhibition of secondary nucleation (Fig. 1). This entails that off-pathway oligomer surfaces are not active in secondary nucleation, in contrast to the obstructed fibril surfaces, suggesting that the surface features of the highly ordered cross-β architecture of amyloid fibrils are essential for secondary nucleation.[48]

We have previously reported inhibition of secondary nucleation by binding of off-pathway

oligomers to amyloid fibril surfaces for Aβ and for lysozyme (ref [27]). The finding that the same applies to αSyn indicates that the interaction between these two distinct protein assembly types might be more the rule than exception. In this context it is interesting to note that there is evidence for delayed fibril formation under conditions of increased off-pathway oligomer formation for further amyloid proteins.[28]

The fact that both homo- and hetero-oligomers inhibit secondary nucleation demonstrates that sequence identity between the fibril-forming and the oligomer-forming protein is not required. This is in contrast to the highly sequence-specific self-assembly at the fibril end during fibril elongation.[49] The oligomers' interaction with fibril surfaces apparently depends on more universal properties of amyloid oligomers. One likely factor is the presence of hydrophobic patches on oligomer surfaces which may interact with ladders of hydrophobic amino acid residues along amyloid fibril surfaces.[50,51] In general, both oligomers and fibrils consists of multiple copies of identical molecules, enabling multivalent interactions of increased stability, potentially involving the rigid cross-β core of amyloid fibrils as well as the less ordered segments that constitute the fuzzy coats of fibrils and oligomers.

The interaction of off-pathway oligomers with fibrils has implications for the distribution and time evolution of the different aggregated species. By inhibiting the growth of fibrils which compete for monomers, off-pathway oligomers promote their own formation and slow down their replacement by amyloid fibrils. In addition, plaques of amyloid fibrils might accumulate off-pathway oligomers and serve as a reservoir of these particularly detrimental species.[39] The observation that hetero-oligomers are amenable to this type of interaction is interesting in the light of reports on interactions and pathological overlap of different amyloid proteins, including αSyn and Aβ.[14–23,48] The data in this study demonstrates that such cross-interactions may also involve the interplay between amyloid fibrils of one protein with off-pathway oligomers of another protein. The interplay between off-pathway oligomers and amyloid fibrils adds another level of complexity to the homo- and hetero-assembly processes of amyloidogenic proteins.

Materials and methods

Protein expression and purification

Expression of αSyn and AS69fus, co-expression of dimAβ and their respective purification was carried out as described previously.[52,27,42] The gene encoding dimAβ included an N-terminal methionine, a first Aβ40 unit, a $(G_4S)_4$ linker, and a second Aβ40 unit.

Preparation of αSyn fibrils

αSyn wildtype fibrils were prepared in 25 mM sodium acetate buffer, 50 mM NaCl, pH 5, while αSyn1–108 fibrils were prepared in 25 mM MOPS, 50 mM NaCl, pH 6.8, both at a protein concentration of 25 μM and in a 1.5 ml reaction vial. 0.03% NaN_3 was added to prevent protein decomposition. To induce fibril formation, the samples were incubated at 37°C over night under shaking conditions at 800 rpm after adding a glass bead.

Preparation of αSyn- and dimAβ oligomers

Off-pathway oligomers were prepared following previously published protocols[35,43,44]: DimAβ oligomers were prepared by dissolving HFIP-treated and lyophilised dimAβ in a small volume of 50 mM NaOH, if necessary facilitated by 1 min of bath sonification. Afterwards, 25 mM MOPS, 50 mM NaCl, pH 6.8, was added before the pH was adjusted with 50 mM HCl, obtaining a final dimAβ concentration of either 10 or 20 μM, depending on the experiment. 0.03% NaN_3 was added to prevent protein decomposition. To induce dimAβ oligomer formation, the samples were incubated at 37°C for about 20 h under quiescent conditions, checked for oligomer formation by AFM and if necessary, left for longer.

αSyn was dialysed over night against water, lyophilized and then resuspended in 25 mM MOPS, 50 mM NaCl, pH 7.4 (since αSyn off-pathway oligomers do not form at pH 5 using the protocol applied here) at a concentration of 12 mg/ml before incubation for 4 h. After centrifugation for 10 min at 16000 rpm, the sample was loaded onto a Superdex 200 increase 10/300 GL SEC column and the oligomer peak was separated from the monomer peak. The oligomers were then concentrated using a spin-concentrator with a cut-off of 3 kDa.

ThT aggregation kinetics

For secondary nucleation assays for wildtype αSyn, αSyn fibrils were sonicated for 3 min in a sonication bath before adding them to a concentration of 100 nM (all concentrations in monomer equivalents) into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3, off-pathway oligomers in varying concentrations, 70 μM freshly thawed αSyn monomer and 50 mM sodium acetate buffer, 50 mM NaCl, pH 5. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 5 min using the BMG Reader Control software (version 5.40).

For αSyn1–108, in addition to the above-mentioned assay, a shaking assay was employed.

For this, 100 nM fibrils were added into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3, off-pathway oligomers, 70 μM freshly thawed αSyn monomer, 50 mM MOPS, 50 mM NaCl, pH 6.8, and a glass bead. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 100 s while shaking at 600 rpm (double-orbital) using the BMG Reader Control software (version 5.40).

For the elongation assay, 2.5 μM fibrils were added after 1 s probe sonification at an amplitude of 10% into the wells of a 96-well low-binding plate (Greiner) with 25 mM ThT, 0.03% NaN_3, off-pathway oligomers in varying concentrations, 25 μM freshly thawed αSyn monomer, 50 mM MOPS, 50 mM NaCl, pH 6.8. The plate was put in a BMG FluoStar Omega and data points were collected at 37°C every 100 s under quiescent conditions.

To determine the lag-times, the linear part of the sigmoidal curves was fitted through the point of steepest ascend and extrapolated to the baseline.

Fibril-oligomer coincubation

5 μM αsyn1–108 fibrils and 5 μM preformed dimAβ oligomers were combined in 50 μL 25 mM MOPS, 50 mM NaCl, pH 6.8, and agitated at 25°C for 10 min before centrifugation at 8000xg for 5 min. The supernatant was then transferred into another vial and the translucent pellet was resuspended in 20 μL buffer, yielding two samples. For the control samples, only the dimAβ oligomers were agitated and centrifuged in parallel, yielding two samples as well.

SDS–polyacrylamide gel electrophoresis

The coincubation samples were analyzed qualitatively by SDS–PAGE and colloidal Coomassie staining. For this purpose, 16 μL of each sample was mixed with 4 μL of 5x sample buffer yielding a concentration of 1x buffer (15% glycerol, 4% SDS, 50 mM Tris–HCl, pH 7.4, 2% β-mercaptoethanol) and 15 μL of each sample was applied onto a 20% Tris/Tricine gel containing a 5.6% stacking gel prepared according to standard protocols. Electrophoresis was performed at a constant current of 40 mA per gel.

Atomic force microscopy

5 μl of the samples were taken, applied onto freshly cleaved muscovite mica, and left to incubate under high humidity for 15 min before carefully washing with 200 μl MQ water and drying under a stream of filtered N_2 gas. Imaging was performed in intermittent contact mode (AC mode) in a JPK Nano Wizard 3 atomic force microscope (JPK, Berlin) with a silicon cantilever and silicon tip (OMCL-AC160TS-R3, Olympus) with a typical tip radius of 9 ± 2 nm, a force constant of 26 N/m, and resonance frequency around 280 kHz. Image processing was done using the JPK DP Data Processing Software (version spm-5.0.84). For the presented height profiles, a polynomial fit was subtracted from each scan line first independently and then using limited data range.

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Wolfgang Hoyer reports financial support was provided by European Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

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3

Discussion and Outlook

The endo-lysosomal system might be an important source for Aβ oligomers

It has been established that the main neurotoxic Aβ structure are its oligomers but at the same time, the formation of oligomers has mostly been described as concentration-dependent with an unphysiologically high threshold *in vitro* (Jan et al., 2010; Lambert et al., 1998). This was consistently one of the main reservations to the importance of oligomers (Morinaga et al., 2010) . In chapter 1 we were able to show that the endo-lysosomal system is an environment in which Aβ not only assembles but is also much more likely to form stable oligomeric structures. We thereby bridge the gap between *in vitro* and *in vivo* considerations, giving rise to an extended model of APP processing and Aβ oligomerization (see fig. 3.1).

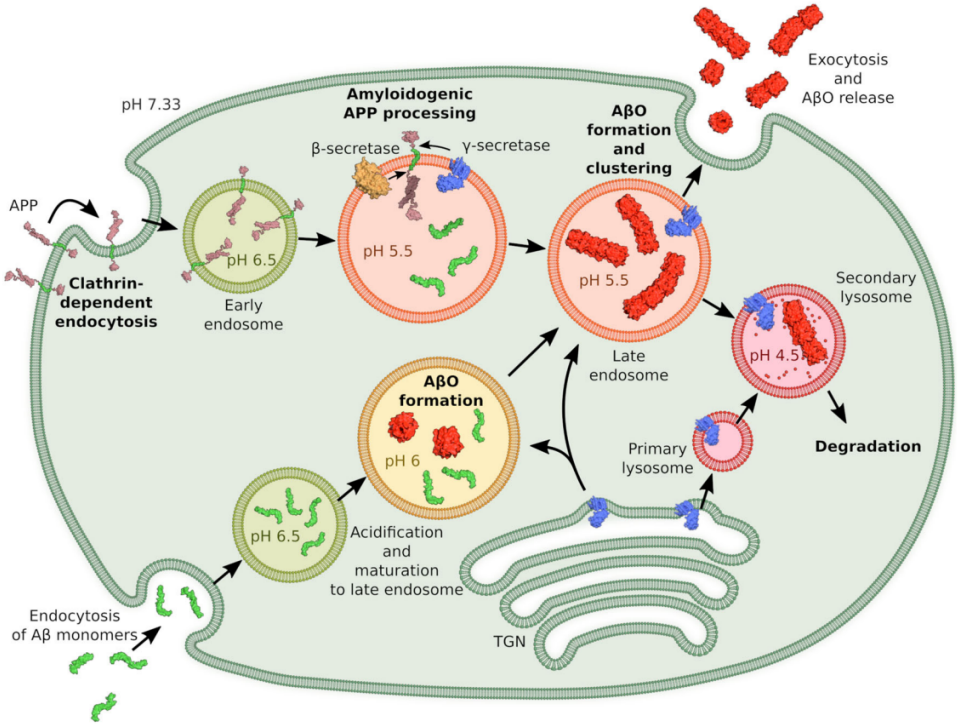

Figure 3.1: Depiction of intracellular APP processing, Aβ uptake, and Aβ oligomer formation as hypothesized in Schützmann et al. (2021).

By utilizing the synthetic Aβ dimer dimAβ, which is an excellent model for Aβ oligomers, we could show that oligomerization kinetics were highly dependent on the pH of the buffer.

The oligomerization rate constant increases around four orders of magnitude from neutral to acidic pH, forming oligomers much faster and also more reproducible at lower concentrations. In addition to this, the second phase of the characteristic biphasic kinetic profile stops appearing at pH 6.4 and lower, corresponding to no aggregation happening. This is likely due to the off-pathway oligomers being stabilized by the acidic pH and not releasing the monomer needed for aggregation. As seen under the AFM, the shape and size of the oligomers changes as well: The curvilinear oligomers that at neutral pH are dispersed homogeneously start forming superstructures that become larger the lower the pH. Both the disappearance of the second kinetic phase and this floculation coincide. This behavior was also confirmed for Aβ42, which showed the same tendencies if at higher concentrations.
These findings appeared relevant because there are organelles in which the pH is notably lower than in the surrounding cytoplasm: The endo-lysosomal system, in which the pH can drop to 4.5. This is especially relevant since Aβ has been observed to accumulate in these compartments with its concentration elevated to above 2.5 μM, four orders of magnitude exceeding the extracellular level (Hu et al., 2009). This is in the same range as the lowest concentration for which Aβ42 showed lagfree oligomerization *in vitro* (5.4 µM). This observation is in line with former studies that reported Aβ "aggregation" in the endo-lysosomal compartment, which happened without lag time (Esbjörner et al., 2014). The results from chapter 1 suggest that the authors might have observed oligomerization instead.
The clearance of Aβ oligomers is not only impeded in lysosomes, they are also prone to leak aggregated Aβ species into the cytosol (Soura et al., 2012; Umeda et al., 2011). It was therefore advisable to examine the behavior of the superclusters formed in an acidic environment upon pH increase to simulate this event. We found that while there was no apparent depolymerisation into monomer since the ThT signal remained stable over days, the morphology of the clusters changed: they began releasing smaller oligomers into the surrounding which had the exact shape of the curvilinear structures formed and observed previously at neutral pH. This not only confirmed that the superstructures at acidic pH are composed of individual Aβ oligomers but also gave a hint to what would follow upon lysosomal leakage.
Since the pH shift did not dissolve the oligomers into monomers, the endo-lysosomal system could potentially be a source for the generation of oligomeric species. Chapter 1 supports the leading role of Aβ in the pathogenesis of AD, because the process of oligomerization is upstream independent of tau, but downstream triggers tau missorting. It however makes no statement towards the relative impact of Aβ oligomers in comparison to tau.

Structural comparison of dimAβ and αsyn oligomers

In chapter 1, a first low-resolution structure of the building blocks of dimAβ oligomers was published. This new structure allows for a comparison of Aβ and αsyn oligomers, which is motivated by chapter 2, in which we showed that they can both interact with αsyn fibrils in a similar way.
It was only possible to roughly estimate the size, volume and overall shape due to the heterogeneity of the oligomeric sample, but the achieved resolution of 17Å is comparable to the 18 resp. 19 Å that were reached by Chen et al. (2015). The sizes of the oligomers are in the same order of magnitude: the αsyn oligomers were roughly cylindrical with a volume of 1030 and 404 nm^3 (excluding the cavity), whereas the dimAβ oligomers were estimated to have roughly the shape of a kidney bean with a volume of 154 nm^3 (see fig. 3.2). Considering that the imaged dimAβ oligomers only represented the smallest units in a heterogeneous population, these values are also comparable.

The most obvious difference between them is the pore-like morphology of the αsyn oligomers, which has no real counterpart with dimAβ. This is not unexpected however, since the mechanisms underlying toxicity hypothesized for the oligomers of αsyn and Aβ have different focal points: αsyn oligomers are often thought to impair membrane integrity. This is in line with their size and overall shape because they are large enough to completely penetrate both the cell membrane (30-50 Å in thickness) and the mitochondrial membranes (each 70 Å in thickness) (Nagle and Tristram-Nagle, 2000; Perkins et al., 1997) and can therefore establish uncontrolled contact between cellular compartments. Even though the structure in chapter 1 was captured using the synthetic dimer dimAβ, it will likely have some structural overlap with Aβ oligomers, since they have a comparable effect on neurons. Therefore the kinetically stabilized dimAβ oligomers can provide a closer look at the shape of those formed by Aβ. In contrast to αsyn, Aβ oligomers exert their influence largely by binding to receptors, for which the epitope does not have to be hollow.

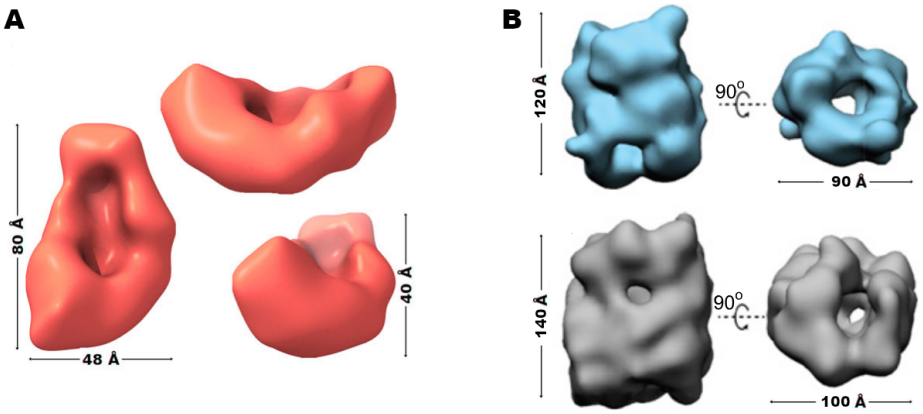

Figure 3.2: Structural comparison of the off-pathway oligomers formed from dimAβ (**A**) and αsyn (**B**) used in chapter 2 with their dimensions. Adapted from Schützmann et al. (2021) and Chen et al. (2015).

While the oligomers' function in relation to toxicity is likely different, their *in vitro* behavior regarding binding to fibrils is similar as shown in chapter 2. This indicates that the presence of the channel in αsyn oligomers is not a factor in this process, but other, shared properties.

The inhibitory effect of off-pathway oligomers crosses sequence-boundaries

It is well-established that off-pathway oligomers inherently inhibit aggregation because they are in equilibrium with monomers but not with fibrils. It has also been shown that off-pathway Aβ oligomers inhibit Aβ aggregation not only via this mechanism but also by directly binding to the fibril surface and thereby masking secondary nucleation sites (Hasecke et al., 2021). In addition to this, in chapter 2 we were able to confirm another example of homoinhibition (αsyn oligomers inhibiting αsyn aggregation), as had also been shown by Yang et al. (2021) and report on an example of heteroinhibition (dimAβ oligomers inhibiting αsyn aggregation).
This result underlines the finding of Hasecke et al. (2021) by completely excluding the possibility of the inhibitory effect being caused by the depletion of the monomer pool. Since the equilibrium between monomers and oligomers that is present in homo-experiments is non existent in this set-up, the mechanism has to be different.
However, it also expands on these findings: Hasecke et al. (2021) showed that the inhibition of the secondary nucleation of Aβ was specific to Aβ itself and to the BRICHOS chaperone

(which is known to inhibit the generation of Aβ42 oligomers (Cohen et al., 2015)), but not to a set of control proteins. Since the function of a chaperone is the interaction with un- or misfolded proteins, this inhibition seems to be within the functional range of the protein. In contrast to this, in chapter 2 we show a case of heteroinhibition that cannot be explained by protein function but also excludes the necessity of a shared sequence for the inhibitory effect to take place. It therefore centers other, shared properties between the protein structures that are able to inhibit secondary nucleation.
The two properties that stand to reason are the size and overall shape of the structures and their hydrophobicity. This is in line with the observation that both properties have a marked influence on oligomer toxicity in combination with each other (Mannini et al., 2014). The overall sizes of both oligomer types are comparable and excluding the channel, the shapes are both compact and ellipsoidal/ cylindrical. The BRICHOS chaperone is more disk-like in structure with a molecular weight of 112.25 kDa (PDB entry 2YAD), which is roughly half or a third of the weight of the αsyn oligomers, but still comparable.
Amyloid oligomers in general seem to have a less dense core and more exposed hydrophobic surface than monomer or fibril (Bolognesi et al., 2010). This feature seems to be connected to the neurotoxicity of oligomers (Kheterpal and Wetzel, 2006; Mannini et al., 2014). In addition to this, concerning hydrophobicity as a driving force in oligomer-fibril interactions, there are several studies of other oligomer interactions that strengthen this argument. For instance, Aβ42 is known to bind the calcium-binding protein calmodulin and cause structural changes to it, driven by hydrophobic interactions (Kim et al., 2023; Salazar et al., 2022). In fact, the amino acid stretch which binds it most strongly is a segment found to not be included in the core of Aβ oligomers and therefore likely being located on their surface (Corbacho et al., 2017). The interaction between Aβ and the islet amyloid polypeptide (IAPP) is also mediated by hydrophobic regions (Andreetto et al., 2010).

Hydrophobicity as a focus point for aggregation modulators: One of the potential avenues for therapeutics against AD are aggregation modulators targeting Aβ. Apart from monoclonal antibodies, the candidates are usually low-molecular structures or (D-) peptides that interfere with one or more aggregation subprocesses by binding one or more structural intermediates and removing them from the equation. The most interesting intermediate to bind are oligomers because of their neurotoxicity. If oligomers bind to fibrils and potentially other surfaces via hydrophobic interactions, the hydrophobicity of interfering compounds should be a focus point. In fact, some developments support this angle, such as more hydrophobic D-peptides showing stronger binding to Aβ species, among them oligomers (Ziehm et al., 2018).

Implications for Pathologies: While most neurodegenerative or systematic amyloid diseases have one or at most two proteins that are thought to be mainly responsible for the pathogenesis (such as αsyn in PD), there are multiple reports of co-pathologies (Hamilton, 2000; Irwin and Hurtig, 2018; Kurata et al., 2007). On the molecular level, Aβ has been found to trigger αsyn aggregation as well worsening neuronal deficits (Köppen et al., 2020; Masliah et al., 2001). In Chapter 2, we show that there also is an interaction on the oligomeric level.

DimAβ proves to be a reliable model for Aβ42 oligomers

DimAβ is an Aβ40 dimer that is covalently linked in a head-to-tail-fashion with a flexible, 20 amino acids long glycine-serine-linker. In Hasecke et al. (2018) it was introduced as a model for Aβ oligomers and was deemed fit for this purpose because 1. it formed oligomeric structures similar to those of Abeta42, 2. it showed kinetic profiles similar to those of Abeta42, even if at

a much smaller concentration and 3. there was evidence to the linker neither interfering with monomeric nor with aggregated forms. In chapter 1, we now also show that the off-pathway oligomers formed by dimAβ can induce tau missorting, show dendritic spine binding, accumulate in the lysosomal system and decrease neuronal activity but are not directly cytotoxic, properties that are known for Aβ42 oligomers. It can be argued therefore that dimAβ behaves like Aβ42 *in vivo*.
This is especially interesting, because the building blocks for this synthetic dimer are Aβ40, which is generally thought of as less toxic. This increased toxicity of dimAβ would therefore likely be due to the kinetic stabilization because of the artificially increased local concentration. Since the difference in the two most common forms of Aβ are only two amino acids that serve to heighten hydrophobicity in Aβ42, the linker might have a similar effect on the oligomer properties.
The only parameter that cannot be probed without reserve using dimAβ is the concentration of Aβ42 needed for certain effects to take place. Naively it is surely possible to just double the concentration of dimAβ oligomers to arrive at a value for Aβ42 oligomers, but this presumes all Aβ42 monomers to be involved in oligomeric structures. This is however far more unlikely than with dimAβ because of the kinetic stabilization and thus the real equivalent concentration of Aβ42 will always be higher than the corresponding dimAβ concentration.
In conclusion, dimAβ is a great model for Aβ42 oligomers, both from a practical and a scientific point of view. It simplifies handling for *in vitro* experiments and provides relatively consistent results and it can also be used for cell culture or even animal experiments to research the effect of Aβ42 on different systems.

4

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