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Mechanistic insights into Aβ-dependent neuronal hyperactivity in an Alzheimer's model

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Abstract

For decades, Alzheimer's disease (AD) research has spent enormous resources to provide better care for patients. The disease is tightly linked to the amyloid-beta (Aβ) peptide which is known to deposit to insoluble plaques over time. These were addressed by clinical studies; however, disappointing results indicate that other, more toxic Aβ species must exist. At the same time, scientists discovered that smaller soluble Aβ assemblies called Aβ oligomers (AβOs) closely correlate with cognitive function, carry neurotoxic potential, and are detectable prior to the formation of plaques. Moreover, AβO-mediated early neuronal hyperactivity was found in transgenic AD mice, which was unexpected since neuronal silencing dominates advanced stages of the disease. The field registers a shift of attention towards soluble AβOs and earlier AD stages with preceding neuronal hyperactivation. Although AβOs were investigated in plenty of toxicity assays, less is known about the neurotoxic potential of specific Aβ aggregates within the heterogeneous group of AβOs. Therefore, this study seeks a systematic analysis testing the hyperactivity-inducing potential of different Aβ species.

For this purpose, I established an in vitro slice assay. For validation, I conducted recent in vivo experiments from the Konnerth laboratory with synthetic Aβ dimers in acute brain slices. However, in contrast to in vivo, synthetic Aβ dimers had initially no effect. Puzzled that acute slices had no spontaneous baseline activity under standard conditions, I pharmacologically raised the activity to in vivo-like levels. Remarkably, this intervention made neurons susceptible to Aβ dimers-induced hyperactivation. By contrast, synthetic Aβ monomers were initially ineffective but obtained neurotoxic potential through oligomerization to AβOs. Further, human-derived Aβ extracted from AD brains containing Aβ of varying sizes exclusively triggered neuronal hyperactivity in slices with ongoing activity. In line with the synthetic preparations, human Aβ dimers caused hyperactivation in contrast to human monomers.

Taken together, these results indicate that the hyperactivating potential depends on the size of the respective Aβ peptide. Furthermore, the data identifies the aggregation of monomers as a crucial event to obtain neurotoxic potential. Finally, the inhibition of Aβ monomer aggregation was disclosed as an auspicious therapeutical target against AD.

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Glossary

(A)CSF	(Artificial) cerebrospinal fluid
(f)MRI	(functional) magnetic resonance imaging
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ADDL	Aβ-derived diffusible ligand
AICD	APP intracellular cytoplasmatic domains
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoE4	Apolipoprotein E ε4 allele
APP	Amyloid precursor protein
Aβ	Amyloid beta
AβO	Aβ oligomer
Aη	Amyloid eta
CA1 (3)	Cornu ammonis area 1 (3)
Ca^{2+}	Calcium
Cal 520 AM	Calmodulin 520 acetoxymethyl
CTF	Carboxy terminal fragments
Da	Dalton
DMSO	Dimethyl sulfoxide
EAAT2	Excitatory amino acid transporter 2
EPSP	Excitatory postsynaptic potential
FAD	Familial Alzheimer's disease
FOV	Field of view
GABA	Gamma-aminobutyric acid
GABAR	Gamma-aminobutyric acid receptor
GECI	Genetically encoded calcium indicator
GLT-1	Glutamate transporter 1

HMW	High-molecular-weight
ID	Immunodepletion
IPSP	Inhibitory postsynaptic potential
LMW	Low-molecular-weight
LTP	Long-term potentiation
MCI	Mild Cognitive Impairment
MWU	Mann-Whitney U test
n	Number of slices
n.s.	Not significant
NA	Numerical aperture
NMDA	N-methyl-D-aspartate
p	P-value
PET	Positron emission tomography
pH	Potential of Hydrogen
PiB	Pittsburgh compound B
PS	Presenilin
ROI	Region of interest
sAPP	Soluble APP
SCs	Schaffer collaterals
SEM	Standard error of the mean
SORL1	Sortilin Related Receptor 1
TBOA	Threo-β-benzyloxyaspartic acid
TPM	Two-photon microscopy
TREM2	Triggering receptor expressed on myeloid cells 2
z	Number of cells

1 Introduction

Alzheimer's disease (AD) is known to be the most common form of dementia. In 1906, the psychiatrist Alois Alzheimer detected insoluble protein aggregates in a human brain (Alzheimer et al 1995, English translation of the original paper from 1907). These aggregates consist of amyloid-beta (Aβ) peptides that play a major role in the development of the disease. Aβ peptides are prone to form aggregates that eventually deposit as Aβ plaques. While researchers have focused on plaques as the main culprit of AD for decades, recent results have revealed that especially aqueously soluble aggregates of 2-50 Aβ peptides (Hayden & Teplow 2013, Finder & Glockshuber 2007), the so-called Aβ oligomers (AβOs), are responsible for the majority of Aβ-related toxicity. However, the exact identity of the toxic aggregates within this group remains enigmatic (Benilova et al 2012).

1.1 Alzheimer's disease

AD has aroused overwhelming interest over the last decades. In 2020, around six million people older than 65 years living in the United States were suffering from AD (Fleming et al 2020), and the number will further grow due to the increasing life expectancy, which is the main risk factor for AD (Armstrong 2019). AD is mostly diagnosed in advanced stages with severe symptoms like memory loss, disorientation, and personality changes (Alzheimer's Association 2022a). Unfortunately, there is no effective therapy for AD to date. In order to reduce the harm to patients and their relatives in the future, it is crucial to understand the mechanisms of Aβ-related pathology in the development of AD.

1.1.1 The role of Aβ in AD

Although AD pathology is characterized by a variety of pathological changes with complex interconnections, it is undisputed that the accumulation of Aβ peptides in the central nervous system plays a highly relevant role.

Aβ generation

Aβ is released by membrane processing of the Amyloid precursor protein (APP) (Haass & Selkoe 2007), which is ubiquitously expressed as a type I integral membrane protein

throughout the brain (Ludewig & Korte 2016). APP is processed by enzymes (so-called secretases) via two different ways, the amyloidogenic pathway, which is critical for the development of AD, and the non-amyloidogenic pathway (**Fig. 1**). The latter is characterized by initial alpha (α)-secretase processing. This secretase is a disintegrin and metalloproteinase (ADAM) that divides APP into two fragments, the extracellular large soluble sAPPα and the membrane-bound αCTF. In the amyloidogenic pathway, an enzyme called beta (β)-secretase cleaves sAPPβ off APP at a different cleavage site, while βCTF remains connected to the plasma membrane. The extension domain of sAPP binds to $GABA_BR1a$ and can modulate synaptic transmission (Rice et al 2019). Furthermore, while researchers have found neuroprotective effects for sAPPα (Furukawa et al 1996), sAPPβ mediates axonal pruning and cell death (Nikolaev et al 2009), suggesting physiological and pathological pathways of Aβ processing. αCTF and βCTF are further processed by gamma (γ)-secretases which contain a catalytic subunit called presenilin. A short protein fragment called p3 (3 kDa) is cleaved off αCTF, whereas the AD relevant Aβ (4 kDa) is cleaved off βCTF. In both cases, γ-secretase processing results in persisting APP intracellular cytoplasmatic domains (AICDs). Remarkably, AICDs may play an important role in regulating the intracellular trafficking of APP (Tamayev et al 2009). Depending on the splicing position of the γ-secretase in the amyloidogenic pathway, the released Aβ peptides vary in length from 37 to 43 amino acids (Siegel et al 2017). Among them, Aβ 1-40 is the most frequent (Karren et al 2011), while Aβ 1-42 mainly mediates neurotoxicity (Jarret et al 1993, Dahlgren et al 2002). After Aβ peptides are cleaved from APP, their hydrophobic character makes them prone to aggregate across intermediate steps into Aβ plaques. Additionally, it has been shown that, apart from α- and β-secretases, eta (η)-secretase processing of APP generates CTF-η fragments and Amyloid η (Aη) (Willem et al 2015).

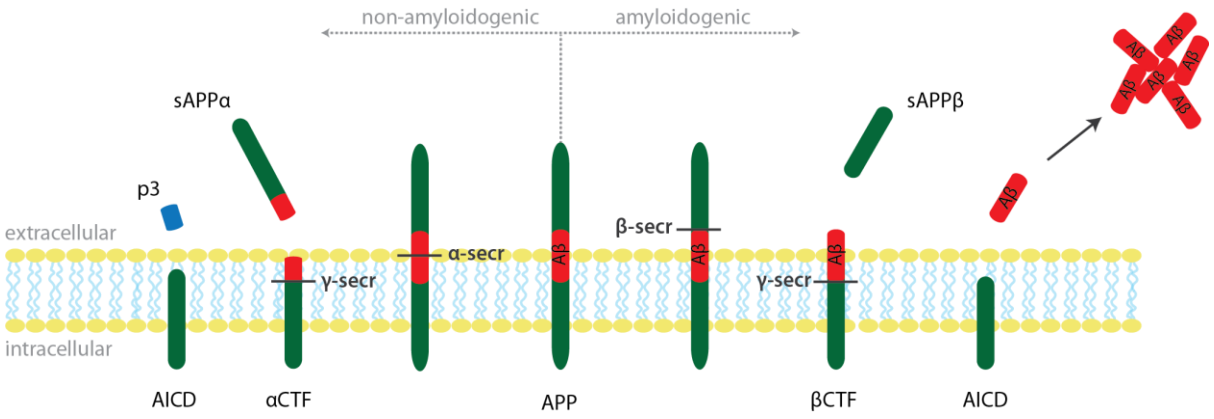

Fig. 1: Schema of APP processing. APP can be cleaved via different pathways. Amyloidogenic processing is mediated by β- and γ-secretases and cause the release of Aβ peptides (right). α- and γ-secretase processing occur in the non-amyloidogenic pathway (left). Note that α-secretase processing prevents the formation of Aβ. APP: Amyloid precursor protein, CTF: C-terminal fragments, sAPP: soluble APP, AICD: Amyloid precursor protein intracellular domain (based on Lichtenthaler 2012).

The amyloid hypothesis of AD

An increase of the total amount of Aβ with a predominance of Aβ 1-42 peptides has been suggested to trigger the formation of Aβ deposits, which is associated with synaptic dysfunction and neuronal injury (Haass & Selkoe 2007). More than 30 years ago, Hardy and Higgins postulated that Aβ plaques cause brain damage resulting in AD dementia, giving rise to the amyloid hypothesis of AD (Hardy & Higgins 1992). This hypothesis is based on numerous studies, which will be discussed briefly.

*Significance of A*β *plaques*

AD is historically linked to Aβ plaques because in 1907, Alois Alzheimer found Aβ plaques in the brain of one of his patients who suffered from a previously unknown disease (Alzheimer et al 1995, English translation of the original paper from 1907). Meanwhile, these AD-related brain changes have been intensively studied and classified neuropathologically. A systematic analysis showed that Aβ plaques are one of the first alterations in the brain (Braak & Braak 1991), and that demented patients exhibit a significant Aβ plaque burden in their cerebellar cortex in contrast to non-demented individuals (Braak et al 1989). Both highlight the importance of Aβ plaques in AD. Furthermore, another study reported a severe neurotoxicity with a focal decrease of neuronal density in the center of Aβ deposits (Urbanc et al 2002). Around the turn of the millennium, scientists established Positron Emission Tomography (PET) imaging to detect Aβ plaques in AD patients in vivo using the radiopharmaceutical tracer Pittsburgh compound B (PiB) (Klunk et al 2004). Studies correlating data from a cerebral PET scan with post-mortem neuropathologic analysis of the Aβ plaque burden reported a tight correlation (Wolk et al 2011, Clark et al 2011). Although a positive Aβ PET scan does not guarantee the diagnosis of AD (Bao et al 2021), patients with a detectable Aβ burden have a significantly increased probability of developing manifest dementia (Okello et al 2009). Notably, the extent of Aβ plaques detected by PiB PET imaging correlates with impaired memory performance (Pike et al 2007), underpinning the significance of Aβ plaques.

Following the discovery of AD-related genetic alterations (discussed in the following section), researchers developed transgenic AD mice forming Aβ deposits (Games et al 1995); these animals revealed memory deficits, increased Aβ levels, and plaque deposition (Hsiao et al 1996). Further, transgenic mice carrying mutations in a humanized APP gene developed plaques with associated neuritic dystrophy (Sturchler-Pierrat et al 1997). Once the major role of plaques turned out to be undisputable, researchers focused on the area surrounding the Aβ plaques and found severe structural alterations such as increased neurite curvature, neuritic dystrophy, and synaptic loss (Serrano-Pozo et al 2011). Notably, the latter represents the major correlate of cognitive decline in AD (Terry et al 1991). In the vicinity of plaques, less mitochondria, disruptions of the mitochondrial membrane as well as fragmentation and dystrophy were observed (Xie, Guan et al 2013). Furthermore, activated microglia, representing tissue specific phagocytes, is located in the vicinity of senile plaques (Serrano-Pozo et al 2011), and reactive astrocytes are also found around senile plaques, which are thought to be activated by microglia (Liddelow et al 2017). Both point to severe neurotoxic potential of Aβ plaques. However, it is still unclear whether astrocytic activation is initiated by AD-related processes and/or may even directly trigger AD pathology (Solito & Sastre 2012). In line with the observed Aβ plaque-associated structural alterations, the corresponding neuronal processing is also impaired because neuronal signals travelling through a plaque are distorted (Knowles et al 1999). Taken together, this evidence points to the critical role of Aβ plaques, which is further supported by the fact that plaque-induced neuritic alterations disappears after anti-Aβ treatment (Lombardo et al 2003).

AD-related genetic alterations

The amyloid hypothesis is strengthened by genetic alterations which were found to cause early-onset familial forms of AD (FAD). In these cases, mutations were detected that lead to an extended generation of Aβ peptides, Aβ plaque formation, and the development of severe early-onset AD pathology. In 1991, scientists detected a point mutation in the APP gene on chromosome 21 in a family suffering from FAD. This mutation caused an amino acid replacement close to the carboxy-end of Aβ (Goate et al 1991). Genomic duplications of the APP gene were identified in five families that caused hereditary early-onset severe AD pathology (Rovelet-Lecrux et al 2006). Moreover, a genetic alteration at codon 673 of the APP gene

causes the substitution of alanine for valine resulting in an increased rate of APP processing through the amyloidogenic pathway. This leads to an elevation of the total Aβ production and abundant deposition of Aβ plaques (Giaccone et al 2010). Additionally, APP mutations close to the β- or γ-secretase cleavage sites were reported to enhance the Aβ production in total and/or cause an isolated shift towards more neurotoxic Aβ 1-42 peptides (Dahlgren et al 2002), whereas mutations that alter the amino acid sequence of Aβ are known to also enhance the plaque forming potential (TCW & Goate 2017). Remarkably, researchers also found a protective mutation against AD in the APP gene of an Icelandic cohort (Jonsson et al 2012). Patients suffering from trisomy 21 — who carry a third APP gene — are doomed to develop AD-related neuropathology (Burger & Vogel 1973, Olson & Shaw 1969) and two-thirds show severe AD-dementia by the age of 72 (TCW & Goate 2017). The third APP copy leads to increased Aβ levels (Wisniewski et al 1985, Mann 1988), even in the brain of an unborn child (Teller et al 1996).

Furthermore, mutations in APP processing enzymes also cause FAD. γ-secretase cleavage is the key step in APP processing that releases Aβ. Presenilin (PS) 1 and 2 represent catalytic subunits of the γ-secretase complex and mutations in the PS1 gene in particular are commonly observed in FAD families (De Strooper 2003). It is known that PS genes are especially affected by missense mutations leading to the exchange of a single amino acid (Selkoe 1996). For instance, a missense mutation in the S182 gene encoding for PS1 was found to cause FAD in 1995 (Sherrington et al 1995). Meanwhile, multiple PS1 alterations such as L435F, P436Q, and P436S mutations have been discovered, but not all PS1 mutations are pathogenic (Heilig et al 2010). By contrast, mutations in PS2 are rare. The first point mutation in the PS2-encoding gene STM2 was found in human from Volga German AD relatives (Levy-Lahad et al 1995). Apart from point mutations, two frameshift mutations in the PS2 gene were discovered in FAD Moroccan patients (El Kadmiri et al 2014).

It remains controversial how a mutated PS gene triggers AD pathology. Mutations selectively increase the Aβ 1-42 level leading to an elevated Aβ 1-42 / Aβ 1-40 ratio, which is known to contribute to the deposition of Aβ plaques (Scheuner et al 1996). A deficit of γ-secretase activity was reported to diminish the generation of Aβ (Herreman et al 2000, De Strooper et al 1998). Following this logic, PS mutations must be gain-of-function mutations causing an extended release of Aβ, however, growing evidence proposes that PS mutations are loss-of-

function mutations. De Strooper suggests that the mutations cause an altered substrate cleavage by the γ-secretase, which may consecutively affect the cleavage of other peptides in the catalytic center. Consequently, this leads to the formation of fewer, but longer Aβ peptides (De Strooper 2007) that may also explain why PS mutations correlate with elevated levels of Aβ 1-42 but decreased Aβ 1-40 (Kumar-Singh et al 2006, Qi et al 2003).

As mentioned, APP and PS mutations are mainly responsible for FAD. However, FAD represents only a small fraction of AD prevalence, as more than 95 % are spontaneous late-onset cases. While age remains the major risk factor (Guerreiro & Bras 2015), the genetic background has also been reported to determine the risk for late-onset AD (Gatz et al 2006). Meanwhile, several genes are known to be associated with this common form of dementia and were previously reviewed in detail (Bellenguez et al 2020). Among them, the ε4 allele of the Apolipoprotein E (ApoE4) was identified as a leading risk factor in contrast to the ε2 and the ε3 alleles (Farrer et al 1997). ApoE4 carriers have a 3 - 15 fold elevated AD incidence depending on the zygosity of the respective alleles (Husain et al 2021). Researchers found a strong association of ApoE4 and the Aβ plaque burden (Schmechel et al 1993). Although the exact pathogenic mechanism remains an enigma, data suggests alterations of the Aβ aggregation and clearance (Kim et al 2009). ApoE4 was found to bind Aβ (Strittmatter et al 1993) and form complexes (Näslund et al 1995); furthermore, it promotes the formation of large Aβ filaments (Ma et al 1994). Therefore, Aβ recycling may be impaired because ApoE4 may shield Aβ from clearance (Verghese et al 2013).

Additionally, variants in the triggering receptor expressed on myeloid cells 2 (TREM2) are associated with an elevated risk of developing late-onset AD (Guerreiro et al 2012, Jonsson et al 2013). Mechanistically, TREM2 controls pathways that regulate phagocytosis and is involved in debris clearance, probably Aβ, in the central nervous system (Takahashi et al 2007). Consequently, impaired receptor activity may result in a gradual increase of the Aβ burden (Guerreiro et al 2012) that may facilitate late-onset AD. Lastly, the risk for late-onset AD is increased by specific variants in the Sortilin Related Receptor 1 (SORL1) gene. SORL1 is part of the APP processing pathway and strengthens non-amyloidogenic pathways (Andersen et al 2005). Thus, the absence or hypoactivity of SORL1 may shift APP towards β- and γ-secretase processing resulting in more Aβ (Rogaeva et al 2007, Dodson et al 2006).

Together, genetic alterations that cause FAD or enable spontaneous late-onset AD are heterogenic, but they all cause an increase of Aβ levels in the brain. Therefore, they provide evidence supporting the amyloid hypothesis that Aβ mainly contributes to AD-related pathology.

1.1.2 The cascade of Aβ aggregation

After its cleavage from APP, the hydrophobic character of the monomeric Aβ peptide makes them prone to aggregate. Aβ monomers — mainly Aβ 1-40 and Aβ 1-42 — form soluble assemblies and, depending on the number of Aβ peptides, these products are called dimers, trimers, and lower- and higher-molecular-weight oligomers. With growing size, aggregates become less soluble and form the so-called (proto)fibrils. Lastly, multiple large aggregates connect to form deposited Aβ plaques. Between intermediates, there is a complex bidirectional equilibrium which is not yet fully understood (Benilova et al 2012).

From monomers to plaques

Aβ 1-40 and Aβ 1-42 monomers are amphipathic peptides (Chen et al 2017). Because of polar amino acids, the N-terminal part of the peptide is hydrophilic while the C-terminal end consists of aliphatic hydrophobic amino acids. In contrast to Aβ 1-40, Aβ 1-42 monomers have two additional hydrophobic amino acids at the C-terminal end. This structural difference provokes distinct aggregation pathways. Aβ 1-42 rapidly form paranuclei representing a circular array of penta-/hexamers that further assemble to form larger Aβ species. Aβ 1-40 monomers are more likely to be in a state of equilibrium with dimers and trimers (Bitan et al 2003). Consistently, synthetic Aβ 1-40 monomers form stable dimers at low concentrations (Garzon-Rodriguez et al 1997); one research group estimated that a monomer concentration of 10 – 40 µM is required for Aβ assembly (Harper & Landsbury 1997). Although Aβ 1-42 is more prone to oligomerize than the shorter Aβ peptide, and the assembly is faster resulting in larger AβOs, Aβ 1-40 can also transform to higher-molecular-weights after a prolonged incubation period (Blackley et al 2000, Goldsbury et al 2000).

In cell cultures, Aβ forms dimers at much lower concentrations (Walsh et al 2000). Notably, most of Aβ aggregation experiments were conducted in vitro, but in vivo conditions turned out to affect the aggregation of Aβ (Finder & Glockshuber 2007, Owen et al 2019). Since various Aβ sizes were found in human brains, numerous AβOs with a molecular mass ranging

over a broad spectrum must coexist in an equilibrium state in vivo (Roher et al 1996, Kuo et al 1996). Numerous higher-molecular-weight AβOs of up to 50 Aβ subunits and different lower-molecular-weight AβOs such as 17 to 42 kDa Aβ-derived diffusible ligands (ADDLs) have already been described (Finder & Glockshuber 2007, Benilova et al 2012). ADDLS were extracted from soluble AD brain extracts (Klein et al 2004) and can also be produced in vitro by incubating Aβ 1-42 monomers (Lambert et al 1998). At last, an Aβ dodecamer with a molecular mass of 56 kDa was isolated from transgenic AD mice (Lesné et al 2006, note that editors of the journal have been alluded to figure manipulation (Piller 2022), the allegations are still under investigation), while a water-soluble Aβ globulomer with 60 kDa has been identified in human brain samples (Barghorn et al 2005).

Fibrillogenesis of Aβ occurs in a prefinal step of Aβ aggregation as the dissolubility of respective Aβ aggregates declines with growing molecular mass and the assemblies exhibit distinct secondary protein structures such as β-sheets. Protofibrils have been detected as precursors of Aβ fibrils (Walsh et al 1997) and are in a dynamic equilibrium with AβOs (Walsh et al 1999). They undergo structural reorganisation and form insoluble Aβ fibrils that consist of a regular β-sheet structure (Chen et al 2017). Finally, the fibrils assemble into large Aβ plaques, which are the histopathological hallmark of AD. It is most likely that Aβ species of different sizes are in an equilibrium state simultaneously. Aβ monomers, aqueously smaller and larger AβOs, and insoluble fibrils and plaques coexist and might undergo interconversion. Explicitly, there are no data proving that the aggregation of Aβ to larger species is irreversible (Benilova et al 2012).

1.1.3 Aβ clearance

According to the amyloid hypothesis, the accumulation of Aβ triggers brain damage. Thereby, the Aβ burden can increase because (i) more Aβ is released and/or (ii) less Aβ is eliminated by clearance mechanisms. In fact, FAD is typically characterized by a pronounced secretion of Aβ, while the more frequent sporadic AD cases mainly display impaired Aβ clearance (Mawuenyega et al 2010, De Leon et al 2017, Li et al 2022, Selkoe & Hardy 2016).

Several non-enzymatic and enzymatic pathways are involved in Aβ clearance (Yoon & Jo 2012), mainly mediated by microglia and astrocytes. On the one hand, they produce Aβ-degrading enzymes such as neprilysin to clear Aβ from the brain enzymatically

(Ries & Sastre 2016). On the other, they clear Aβ via phagocytosis. The microglia represents the primary immune cells of the central nervous system and exhibits phagocytic capabilities (Shigematsu et al 1992). Once activated by Aβ (Maezawa et al 2011), the microglia phagocytose Aβ (Rogers et al 2002), which suggests a reliable mechanism of keeping Aβ levels low in the brain. Moreover, astrocytes also mediate the clearance of Aβ (Wyss-Coray et al 2003). Cultured human astrocytes bind Aβ for internalization (Nielsen et al 2009), mainly through scavenger receptors such as the low-density lipoprotein receptor-related protein 1 (Yoon & Jo 2012). Since they are part of the blood brain barrier, astrocytes mediate the transport of Aβ from the potentially vulnerable central to the peripheral compartment (Wildsmith et al 2013).

Over decade, the central nervous system was thought to lack a lymphatic system. The discovery of a central glymphatic system has created overwhelming attention in the field and provided a compelling mechanistic framework of Aβ clearance. Researchers discovered that cerebrospinal fluid (CSF) enters the extracellular matrix along paravascular spaces and is again cleared through paravenous pathways (Iliff et al 2012). Additionally, lymphatic vessels associated with the venous sinuses were found to transport fluid and cells from the brain to peripheral lymph nodes (Louveau et al 2015, Albayram et al 2022). These findings offer an additional mechanism, with which toxic Aβ is cleared from the brain. Consistently, one study demonstrated that the disruption of the lymphatic vessel system results in an elevated Aβ burden in the brain (Da Mesquita et al 2018).

Within the context of Aβ clearance, it is important to note that sleep disturbance is frequently observed in AD. For instance, sleep deprivation has been associated with an increased risk of late-onset dementia (Sabia et al 2021), but a sufficient explanation had yet to be found. Though, the Aβ-clearing glymphatic system now provides a mechanistic model for how sleep and AD are interconnected. Scientists discovered that the clearance from the central nervous system increases during sleep and reported a gain of 60 % of the interstitial space with a pronounced exchange of pericellular fluid with CSF (Xie, Kang et al 2013). This indicates that toxic Aβ is extensively removed from the brain during sleep and that, conversely, sleep disturbance impairs Aβ clearance resulting in growing Aβ concentrations in the brain.

1.2 Characteristics of early AD dysfunction – a shift of attention

Historically, Aβ plaques were deemed to be the main trigger of AD-related brain pathology. However, the growing evidence in AD research triggered a shift of attention towards soluble AβOs that occur prior to plaques and mediate synaptic loss and neuritic dystrophy, among other things (Selkoe & Hardy 2016). In the following, I will give a brief overview of this transformation.

1.2.1 From insoluble plaques to soluble Aβ

The problem with plaques

Although the Aβ hypothesis had been supported by multiple lines of evidence over several decades, there have also been conflicting findings. Notably, the Aβ plaque burden correlates neither with cognitive decline (Nelson et al 2012, Hardy & Selkoe 2002), nor with synaptic loss (Chen et al 2017). Moreover, not all AD patients exhibit a significant plaque burden; a group had almost no detectable plaques (Serrano-Pozo et al 2014, Monsell et al 2015). Conversely, normal cognitive function can be observed despite a substantial amount of plaques (Zolochevska & Taglialatela 2016, Mormino & Papp 2018). Further doubts about the significance of plaques in AD came from Phase 3 clinical studies. First, aducanumab was shown to reduce the Aβ plaque burden in transgenic mice (Sevigny et al 2016), however, the clinical benefit remains controversial (Knopman et al 2021a). Second, the use of donanemab to target a modified form of Aβ deposits lowered Aβ essentially to zero (Knopman et al 2021b) but had no impact on secondary outcomes (Mintun et al 2021). These results suggest that further Aβ species must play a pivotal role in AD because Aβ plaques alone are not sufficient to explain the entire pathology.

Preclinical AD stages

For decades, researchers had assumed that plaques and impaired cognition are tightly linked to each other. Though, growing evidence has since shown that preclinical AD stages with specific alterations but without a severe clinic and Aβ plaques in fact exist (Morris 2005). The term “Mild Cognitive Impairment” (MCI) is used to describe this pre-dementia phase of cognitive decline, which is characterized by initial cognitive complaints that do not yet affect the

independent performance in life's daily activities (Alzheimer's Association 2022b) but progresses to severe dementia in the majority of cases (Yaffe et al 2006). AD-specific alterations, for instance hippocampal atrophy, are already detectable in MCI patients (Dickerson et al 2001) and predict the likelihood of progressive dementia (De Leon et al 1989).

Furthermore, in biomarker studies that rely on the assumption that Aβ is a sensitive biomarker for preclinical AD stages, researchers also discovered early AD-related abnormalities (Dubois et al 2016). The Aβ 1-42 / Aβ 1-40 ratio in the blood was shown to be decreased in preclinical AD stages (Janelidze et al 2016), which was also associated with a higher risk for AD (Graff-Radford et al 2007). In line, plasma Aβ 1-42 is significantly decreased in the stage of MCI and the Aβ 1-42 concentration in the blood changes dynamically over time from cognitively normal to AD-demented individuals (Park et al 2022). This indicates that Aβ in the blood is a versatile biomarker for the detection of MCI patients and AD progression. Additionally, alterations of blood Aβ 1-42 correlate with changes in the respective concentration in CSF (Park et al 2022). In the CSF of AD patients, researchers detected reduced levels of Aβ 1-42 (Andreasen et al 1999a), more data suggested that CSF levels of Aβ 1-42 are valuable in distinguishing AD from a control group (Paterson et at 2018). In addition, measuring Aβ in CSF is sufficient to detect the MCI stage. Studies found reduced levels of Aβ 1-42 in CSF compared to healthy controls, which was a reliable predictor for the development of AD (Andreasen et al 1999b, Hampel et al 2004). Similar to blood biomarkers, the Aβ 1-42 / Aβ 1-40 ratio in CSF was proved to be of eminent importance, which further enhances the validity of biomarker analysis (Baldeiras et al 2018).

Taken together, recent evidence implies a preclinical stage of AD dementia in the absence of severe symptoms but with detectable alterations of Aβ as biomarker with predictive value. Most notably, this stage is not characterized by plaques; their burden is not pronounced compared to healthy controls (Markesbery et al 2006). Therefore, Aβ plaques do not seem to be a major substrate of MCI (Markesbery 2010) and in line, transgenic AD mice revealed discreet behavioral abnormalities already in an early-stage prior to the formation of Aβ plaques (Latif-Hernandez et al 2019). Consequently, this all attenuates the role of Aβ plaques, especially in early stages of AD, and calls for a better understanding of amyloid toxicity.

Soluble Aβ - the new culprit for amyloid toxicity

While the number/area/volume of Aβ plaques do not correlate with cognitive decline and AD-related pathology occurs prior the formation of plaques, the consensus is that more neurotoxic Aβ species must exist. Remarkably, in contrast to plaques, soluble Aβ was found to correlate with impaired cognition (Wang et al 1999, McLean et al 1999). Researchers showed in cell cultures that soluble Aβ is more potent in inducing neurodegeneration than larger Aβ species (Picone et al 2009), and one group discovered a 56 kDa soluble AβO in transgenic AD mice that impaired memory in an early AD stage in the absence of plaques. Therefore, soluble Aβ compromises cognition independently from plaques as a foregoing process (Lesné et al 2006, note that editors of the journal have been alluded to figure manipulation (Piller 2022), the allegations are still under investigation). This is further supported by the detection of AD-related neuronal dysfunction in predepositing transgenic AD mice, which could also be induced by soluble Aβ in wild-type mice (Busche et al 2012). Conversely, memory function is unaltered in Aβ plaque-bearing transgenic mice with reduced levels of soluble Aβ (Lesné et al 2008). A so far unknown FAD-related mutation in the APP gene was found in humans causing high levels of soluble Aβ in the absence of plaques (Shimada et al 2011, Inayathullah & Teplow 2011); transgenic animals carrying the respective mutation yielded similar results (Tomiyama et al 2010). The eminent role of soluble Aβ was further supported by the fact that their deleterious effects disappeared after they were neutralized with a specific antibody (Klyubin et al 2005), and cognitive decline was reversed independently from the Aβ plaque burden by passive immunization of transgenic mice against Aβ (Dodart et al 2002).

Soluble AβOs were detected in the immediate vicinity of Aβ plaques forming a halo with a dystrophic microenvironment (Kayed et al 2003, Haass & Selkoe 2007, Sanchez-Varo et al 2021, Koffie et al 2009). This spatial association is not coincidental since insoluble Aβ may act as an inactive reservoir forming an equilibrium state with preceding more toxic and smaller Aβ substrates (Hardy & Selkoe 2002). This assumption is supported by data showing that human Aβ plaques have no effect as long as they are not dissolved to release dimers (Shankar et al 2008). Vice versa, scientists even assume that the formation of plaques is an escape process, which is triggered by the appearance of severely toxic AβOs in the brain (Carrotta et al 2005). In line, an animal model with Aβ plaques reveals no memory deficits during an episode of a decreased AβO concentration, which occurrs during pronounced

plaque formation (Lesné et al 2008). Another group found elevated levels of AβOs in AD patients with Aβ plaques compared to non-demented patients with a comparable plaque burden (Esparza et al 2013). Remarkably, both studies indicate that toxic AβOs directly undergo neutralization through deposition, suggesting that Aβ plaques consist of shielded toxic Aβ (Yang et al 2017). Consistent with this, no AβO-induced plaque-surrounding neuritic pathologies were detected in cognitively healthy humans who carry a large Aβ plaques burden (Hardy & Selkoe 2002). Together with the fact that the level of soluble Aβ distinguishes AD brains from normal aging (Wang et al 1999), it is considered to be safe that soluble AβOs mediate pathological changes (around plaques) and are more critical for AD-related pathology than insoluble plaques (Haass & Selkoe 2007).

1.2.2 Soluble Aβ oligomers

Structural alterations

As mentioned, soluble AβOs represent Aβ monomer assemblies. The term "oligomer" is used for a broad spectrum of Aβ aggregate sizes. One group reviewed that AβOs range from less than 10 kDa to more than 100 kDa in weight including dimers, trimers, and larger oligomers (Sakono & Zako 2010). AβOs are classified as low-molecular- (LMW) and high-molecular-weight (HMW) (**Fig. 2**), e.g., at the cutoff of 50 kDa.

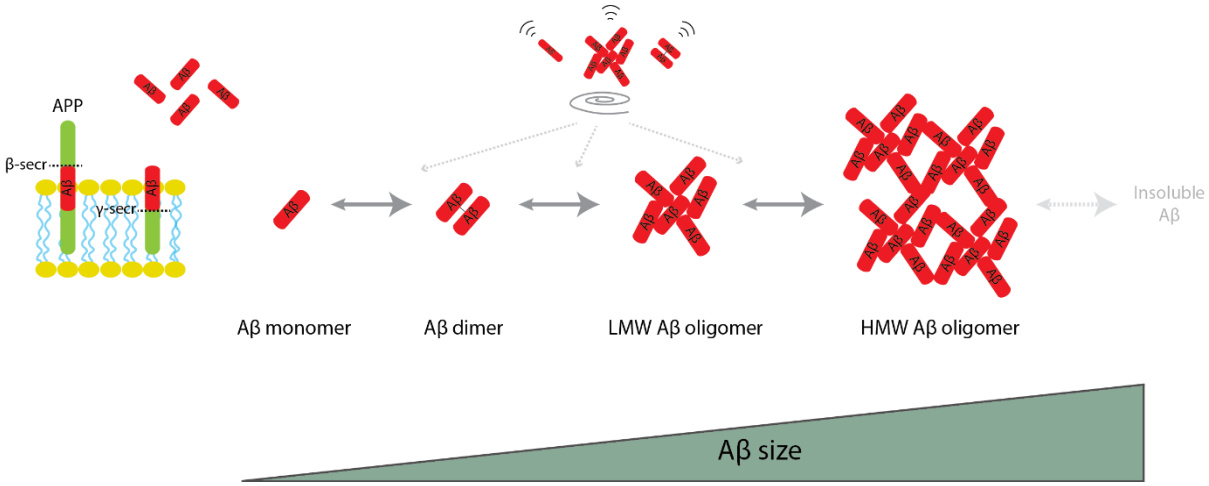

Fig 2: Aβ aggregation. Membrane-bound Amyloid precursor protein (APP) is processed by β- and γ-secretases and monomeric Aβ is released into the extracellular space. Aβ monomers tend to assemble into smaller and larger oligomers. LMW: low-molecular-weight, HMW: high-molecular-weight.

Human-derived Aβ dimers, the smallest of the LMW AβOs, were shown to induce neuronal damage (Shankar et al 2008), small AβOs cause synapse- and spine loss (Shankar et al 2007) — it is widely accepted that dendritic spine loss strongly correlates with cognitive function (Terry et al 1991, DeKosky et al 1996) —, and oligomeric Aβ also triggers hippocampal cell death (Kudo et al 2012) and promotes apoptosis in cell cultures (Guglielmotto et al 2014). Furthermore, AβOs affect spine morphology, alter the composition of the spine cytoskeleton (Lacor et al 2007) and also affect astrocytes apart from neurons. It was shown that Aβ perturbs astrocytic signalling pathways, induces astrogliosis, and lowers the concentration of astrocytic glutamate transporters (Cline et al 2018). Together, AβOs mediate severe neurotoxicity that yields neuronal cell death, cortical atrophy, and cognitive decline in AD.

Functional alterations

Apart from the respective structural alterations, impaired cognitive function is also attributable to AβOs and can be studied with behavioral assays (Puzzo et al 2014). Intracerebroventricularly-injected AβOs obliterate an initially learned behavior in rats (Cleary et al 2005); further, six-month-old transgenic AD mice with already a fraction of triton-soluble Aβ and no plaques (Cohen et al 2013) reveals navigational deficits in the Morris water maze task (Rorabaugh et al 2017), which is a widely used method in behavioral neuroscience (D'Hooge & De Deyn 2001) and AD research. Memory performance is also useful for measuring AD-related cognitive deficits. One study showed that AβOs injected intracerebroventricularly potently impair recognition memory and memory consolidation, whereas the retrieval of consolidated information is unaffected (Balducci et al 2010). This evidence overlaps with data showing that patients with early dementia have deficits in learning but less in remembering (Greene et al 1996). Additionally, long-term potentiation (LTP) experiments provided more data. LTP is an electrophysiological correlate of synaptic plasticity and represents a model for memory and learning (Bliss & Collingridge 1993) that is commonly investigated in AD (Selkoe 2002). It embodies the strengthening of a synaptic connection as a response to repetitive stimulation, resulting in a relatively larger postsynaptic potential induced by a certain unchanged presynaptic signal (Bliss & Collingridge 1993, Cooke & Bliss 2006). LTP is impaired in transgenic AD mice (Selkoe 2002) and human-derived (Walsh et al 2002) as well as naturally secreted AβOs (Klyubin et al 2005, Lei et al 2016) were shown to potently inhibit

hippocampal LTP. In contrast, human-derived Aβ plaques have no effect on LTP induction (Shankar et al 2008), which again underlines the dominating role of AβOs.

AβOs were found to cause membrane depolarization in cell cultures (Morkuniene et al 2015). This is in line with the clinical observation that AD patients carry an increased risk for epileptic seizures (Palop & Mucke 2009), which are already detectable in early AD stages (Vossel et al 2013). Additionally, cognition declines faster in patients with subclinical epileptiform activity compared to patients with normal activity levels (Vossel et al 2016). It seems that AβOs affect neuronal activity and, indeed, human-derived AβOs increase excitatory (E) glutamatergic and decrease inhibitory (I) GABAergic (γ-Aminobutyric acid) signals in hippocampal neurons. This results in a higher E/I ratio (Wang et al 2017) and contributes to impaired cognition in AD (Li & Selkoe 2020). Although GABAergic inhibitory interneurons were initially suggested to be resistant to Aβ toxicity (Pike & Cotman 1993), their dysfunction was found to contribute to AD development (Palop & Mucke 2016). Changes in inhibitory interneurons in transgenic mice and AD patients cause a reduced gamma oscillatory network activity (Verret et al 2012), which is known to play a crucial role in cognitive function and memory (Traikapi & Konstantinou 2021). Interestingly, altered network oscillation in transgenic AD mice appears before Aβ accumulation (Goutagny et al 2013). Iaccarino and colleagues measured reduced gamma oscillation activity prior to plaque deposition and cognitive decline in AD mice and imitating this frequency by using optogenetics potently reduces Aβ levels (Iaccarino et al 2016). In addition to lessen the Aβ burden, the restoration of gamma oscillation also improves memory in AD mice (Martorell et al 2019). This approach has since been tested in clinical trials, for instance, daily, non-invasive gamma sensory stimulation of pre-AD-demented individuals is beneficial in terms of brain alterations and cognitive performance (Chan et al 2022), which can represent a previously overlooked new therapeutic strategy against AD. Since glutamate is the most important excitatory neurotransmitter in the brain, alterations of the well-known AMPA- (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors, as well as altered glutamate homeostasis, are suspected of causing an elevated E/I ratio. Indeed, AβOs enhance the release of presynaptic glutamate resulting in higher excitation in cultured hippocampal neurons (Brito-Moreira et al 2011), and soluble AβOs impair LTP by strongly activating the NMDA receptors (Li et al 2011). Further, a

post-mortem study with autoptic hippocampi from AD patients revealed changes in the subunit composition of glutamate receptors compared to healthy controls (Marcello et al 2012).

Accumulating evidence indicates that AβOs induce structural and functional AD-related pathologies. In the following, I will mainly focus on the AβO-induced disruption of the E/I balance, which causes neuronal dysfunction and is suspected to play a major role in early AD (Hector & Brouillette 2020).

1.2.3 Neuronal dysfunction in AD

A large body of data had suggested neuronal hypoactivity in AD patients. First, patients with senile AD dementia displayed brain hypometabolism in PET scans indicating a reduced neuronal activity (Ferris et al 1980, Johnson et al 2012). Second, AD patients had less activation of the hippocampal formation during a memory encoding task in functional magnetic resonance imaging (fMRI) measuring the blood oxygen level-dependent MRI signal (Sperling et al 2003). This signal correlates with local field potentials and grossly follows neuronal activity (Logothetis et al 2001). Third, AD patients showed lower frequencies and a reduction of complex activity patterns in the electroencephalogram (Jeong 2004). Overall, the loss of activity was assumed as one AD-typical phenomenon being responsible for cognitive decline. However, these methods generate (functional) data from brain areas and lack of cellular resolution, which can in turn be achieved with two-photon microscopy (TPM) in mice.

Using TPM, researchers from the Konnerth laboratory surprisingly found clusters of neurons with increased spontaneous activity levels in the vicinity of plaques in transgenic AD mice in vivo (Busche et al 2008), which in principle corresponds to the Aβ-induced elevated E/I ratio in hippocampal neurons (Wang et al 2017). Hyperactivity was further found in the hippocampus of young transgenic AD mice without pre-existing Aβ deposits (Busche et al 2012). Remarkably, the experiments disclose a tight correlation between AβOs and neuronal hyperactivity: normal activity levels are restored after γ-secretase inhibition lowered soluble Aβ levels and the local application of small AβOs directly triggers neuronal hyperactivity in wild-type mice (Busche et al 2012). As mentioned in chapter 1.2.1, AβOs form a halo around plaques within the area of hyperactive cells (Sanchez-Varo et al 2021). Moreover, a PET imaging study found that the highest glucose uptake in the brain is near those thioflavin-positive Aβ plaques (Poisnel et al 2012), which reflects pronounced neuronal activity levels in the respective area

(Lundgaard et al 2015). Because of this spatial association, AβOs are expected to directly cause neuronal dysfunction.

In addition, a fraction of hypoactive cells was also reported (Busche et al 2008). Thus, there might be a switch from hyper- to hypoactivity with disease progression: in young transgenic AD animals, hippocampal neurons become hyperactive before Aβ plaques have formed. However, with a delay of months, the number of hypoactive neurons start to grow (Busche et al 2012). Consistently, hippocampal neuronal hyperexcitability was electrophysiologically recorded in a mouse model of AD, which was strongly linked to structural alterations (Šišková et al 2014), and a strong association between increased neuronal excitability and neuronal hyperactivity was likewise observed in a rat model (Sosulina et al 2021). Evidence from fMRI studies also supports the concept of early neuronal hyperactivation in prodromal AD cases (Sperling et al 2010). In elderly with MCI, a learning task causes a significant larger hippocampal activation. However, in advanced stages of the disease, the activity is reduced (Dickerson et al 2005), which fits with results from the longitudinal Ca^{2+} experiments. In addition, the extent of hippocampal hyperactivation depends on the amyloid status of the respective patient, and amyloid positivity was shown to be associated with reduced brain volumes (Huijbers et al 2015). Brain activation patterns in human carrying ApoE4 or genetic mutations for FAD were also studied and higher hippocampal activation during memory activation tasks was repeatedly reported (Bookheimer et al 2000, Quiroz et al 2010).

Together, hyperactivity is an early neuronal dysfunction in AD. But what does this mean for cognition? Hyperactive neurons reveal an Aβ-dependent impairment of visual stimuli tuning (Grienberger et al 2012); moreover, almost all transgenic AD mouse models used to study hyperexcitability exhibit an impaired cognitive function (Hector & Brouillette 2020) like longer escape latencies in the water maze task (Keskin et al 2017). Furthermore, pronounced firing rates of hippocampal place cells — neurons whose activity pattern encodes spatial orientation — were recorded in aged cognitively impaired rats with spatial memory deficits (Wilson et al 2005, Robitsek et al 2015). Finally, the restoration of neuronal activity levels with the antiepileptic drug levetiracetam is accompanied with better memory performance in MCI patients who show increased hippocampal activity in contrast to a healthy control group (Bakker et al 2012). Thus, restoring hippocampal activity levels is suggested to improve cognitive function in cognitively affected elderly patients (Koh et al 2010).

In vitro evidence for neuronal hyperactivity

Imaging neuronal activity in vivo is a versatile approach investigating intact neuronal circuits (Stosiek et al 2003), and therefore, experiments to study AD-related neuronal dysfunction were initially conducted in vivo in transgenic animals in the Konnerth laboratory (Busche et al 2008). Even though in vivo imaging can be generally conducted in awake mice — even using head-mounted microscopes in freely behaving animals (Ozbay et al 2018) — the approach is commonly performed under anesthesia. Anesthetics were found to induce structural alterations such as elongated microglia processes (Sun et al 2019) and to affect neuronal activity (Goltstein et al 2015), which is undesirable while investigating neuronal dysfunction. Although the intact organism is most suitably represented by the in vivo approach and it appears obvious to investigate AD-related dysfunctions within this model, there are many parameters that may unintentionally intervene and disturb the experiment. Therefore, in vitro approaches such as acute slice preparations and cell cultures represent alternative approaches to study AD-related neuronal hyperactivity under more controlled experimental conditions.

Aβ and fragments of the peptide induce neuronal excitation in cultured hippocampal neurons and acute slices (Brorson et al 1995, Fogel et al 2014). Moreover, neuronal hyperactivity was also observed in cultures after one day of incubation with AβOs (Ciccone et al 2019) and the inhibition of the degradation of released Aβ enhanced neuronal activity in rat neuronal cultures and slices (Abramov et al 2009). While these experiments demonstrate that in vitro assays are versatile to study neuronal hyperactivity, our goal was to investigate the cellular mechanism of Aβ-dependent neuronal dysfunction. Researchers from the Konnerth laboratory, including myself, discovered that neuronal hyperactivation is dependent on the ongoing baseline activity of the preparation (Zott et al 2019). We initially observed that synthetic Aβ dimers which potently induce neuronal hyperactivity in vivo (**Fig. 3 A**) surprisingly had no effect in freshly prepared acute hippocampal brain slices (**Fig. 3 B**). One obvious difference between the two assays was the ongoing neuronal baseline activity in vivo while pyramidal neurons in vitro were inactive (referred to as “silent”) (**Fig. 3 C-E**). In the following, we pharmacologically induced in vivo-like spontaneous baseline activity in vitro by adding glutamate or potassium and/or bicuculline to the artificial cerebrospinal fluid (ACSF) (Zott et al 2019), which

then made hippocampal slices susceptible for synthetic Aβ dimer-induced neuronal hyperactivation (**Fig. 4**). Thereafter, this discovery paved the way for systematically testing various Aβ species under controlled conditions in vitro.

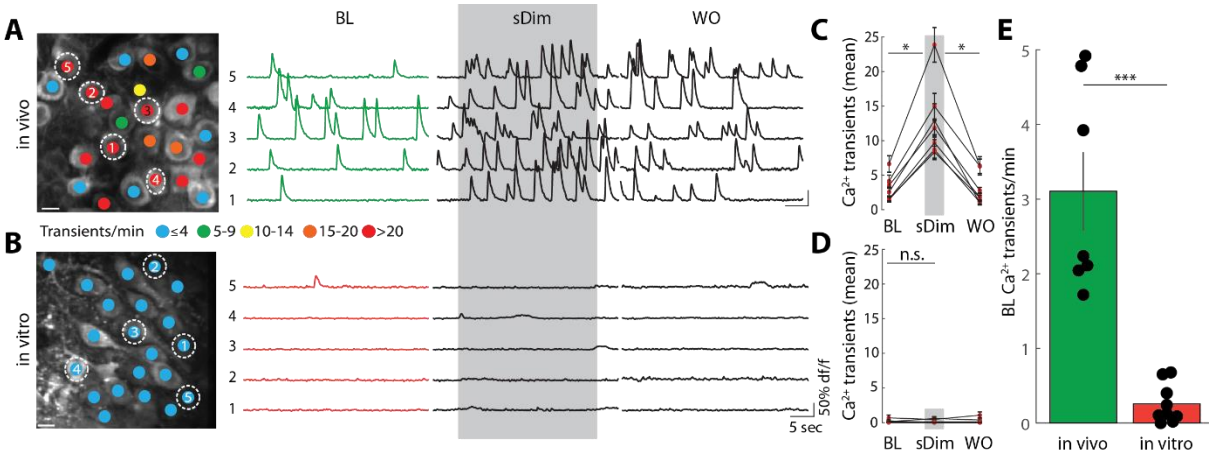

Fig. 3: Synthetic Aβ dimers induce hyperactivity in vivo but not in vitro. A) Two-photon image and Ca^{2+} transients of selected neurons for baseline, synthetic Aβ dimer application (shaded area) and washout for one representative in vivo experiment (from left to right). **B)** Same as A) for one representative in vitro experiment. Colored dots mark neurons and represent the number of Ca^{2+} transients per minute during the Aβ application. Importantly, almost no spontaneous Ca^{2+} transients were detectable in vitro (red lines) in contrast to in vivo (green lines). **C)** Quantitative data for the in vivo experiment shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, Aβ application and washout. Lines connect dots of one single experiment (n = 7). **D)** Same as C) for the in vitro experiment shown in B) (n = 9). **E)** Bar graph of spontaneous baseline activity levels for the in vitro and in vivo experiments shown in C) and D). Each dot represents the average baseline activity per minute for one experiment. Error bars display SEM. Wilcoxon test (C, D) or MWU test (E). * $p < 0.05$, *** $p < 0.001$, n.s. not significant. Scale bars: 5 µm. All figures were adapted from Zott et al 2019 with permission from AAAS.

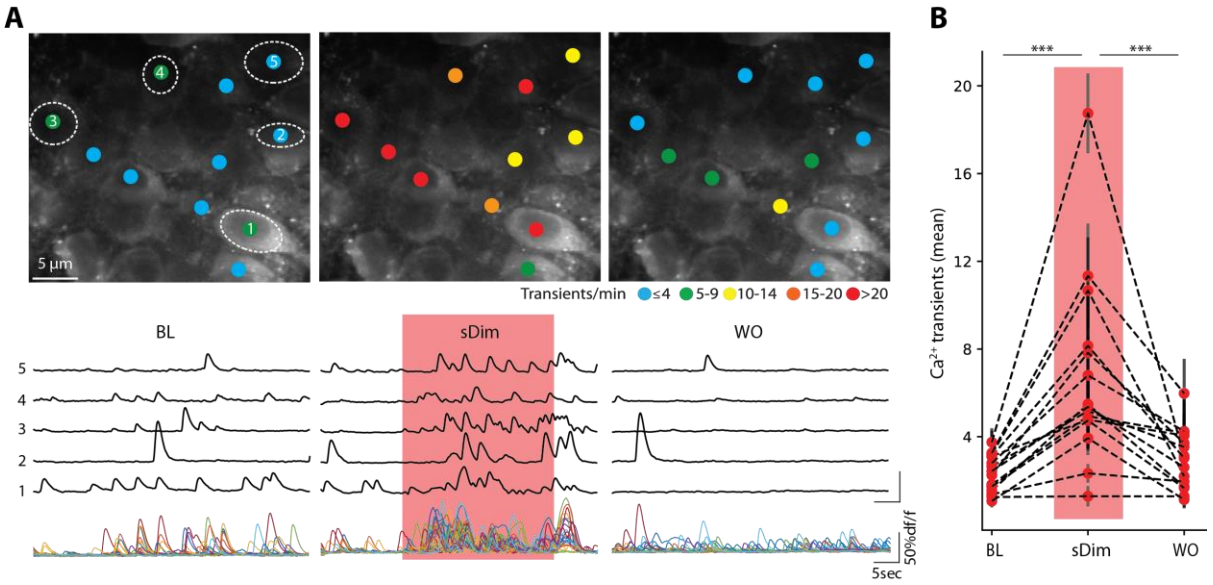

Fig. 4: Creating in vivo-like baseline activity makes hippocampal slices accessible for synthetic Aβ dimer-induced neuronal hyperactivity. A) Two-photon images (up), Ca^{2+} traces of five selected circled cells (middle) and overlayed traces of all cells (z = 27) from the respective experiment (down) for baseline, synthetic Aβ dimer application (shaded area) and washout. Colored dots on neurons represent the number of Ca^{2+} transients per minute during each phase. **B)** Quantitative data for the experiment shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, Aβ application and washout. Lines connect dots of one single experiment (n = 15). Error bars display SEM. MWU test (B). *** $p < 0.001$. Data for (B) was partially adapted from Zott et al 2019 with permission from AAAS.

1.3 Methodological instruction

As described above, TPM can be used to study the effect of Aβ on neurons in the hippocampal Cornu ammonis area 1 (CA1) in acute slices after the induction of in vivo-like baseline activity. In the following, I will give a brief overview of the theory involved.

1.3.1 Activity induction

As previously shown (Zott et al 2019), the neuronal baseline activity must be elevated to in vivo-like levels in order to study the effects of Aβ in vitro. This can be achieved by increasing the extracellular potassium concentration (Florence et al 2009, Bikson et al 2001, Liu et al 2021), which lifts the resting potential to a higher value according to the Nernst equation. Consequently, the probability of cell-firing is increased. The most frequent excitatory neurotransmitter in the brain is glutamate, therefore, adding glutamate to the ACSF solution is another technique to induce spontaneous baseline activity. Although this approach can be used to induce excitotoxicity in slices (Molz et al 2008), it has been shown that a tightly adjusted low dose of glutamate is appropriate to induce activity without severe side effects (Zott et al 2019). CA1 pyramidal neurons receive excitatory and inhibitory synaptic inputs (Megías et al 2001). Consequently, disinhibition with the $GABA_A$ – receptor antagonist bicuculline can also be used to induce in vivo-like activity in vitro by blocking inhibition (Zott et al 2019).

1.3.2 Fluorescent microscopy

Fluorescent microscopy uses the excitation of fluorophores to cause the emission of lower-energy photons that can be detected as signals. When fluorophores are connected to molecules with a specific property (e.g., a calcium-binding protein), fluorescent microscopy can

visualize biological processes. Electrons of the fluorophores are unexcited at their ground state, which represents the lowest and most stable energy level. For fluorescence to occur, a fluorophore must absorb energy to reach an excited state. Subsequently, the excited electrons return to the ground state due to the natural tendency to return to the lowest energy level. To achieve this, the fluorophore emits a photon slightly longer than the excitation wavelength in order to lose excess energy (Lichtman & Conchello 2005). This fluorescent emission can be separated from the excitation light with a dichroic mirror and detected with appropriate devices. For example, the wild-type green fluorescent protein mainly absorbs blue light with a wavelength of 395 nm and emits green light with a wavelength of 510 nm (Remington 2011). A basic technique of fluorescent imaging represents widefield microscopy. The whole sample is exposed to the excitation light at once and consequently, fluorescence is emitted from the entire sample simultaneously. Therefore, the spatial resolution is relatively low compared to other techniques, which will be briefly discussed below.

1.3.3 Single-photon vs. two-photon microscopy

In contrast to widefield imaging, confocal single-photon microscopy utilizes focused single-pixel illumination. Using fast oscillating mirrors, a laser beam is guided across the region of interest (ROI). For every pixel, fluorophores must be excited and the emission has to be detected rapidly before a new excitation cycle begins. Confocal microscopy is the method of choice for studying superficial structures. One excitation photon carries enough energy to excite a fluorophore resulting in a coniform shape of excitation. However, this means that fluorophores are excited not only in the focal plane, but also outside of the focal plane (Merchant et al 2005). In order to obtain a sharp image, the artificial emission from outside the focus must be separated from the signal. This can be achieved by using a pinhole that eliminates photons that do not originate in the focal plane (Elliott 2020) (**Fig. 5 A**). Unfortunately, the pinhole additionally eliminates scattered photons from the focal plane; consequently, most of the emitted photons are eliminated by the pinhole and the remaining light is rather dim, which necessitates amplification by a photomultiplier tube for detection. Another approach to strengthen the signal is to increase the laser intensity, but this is in turn limited by photodamaging and bleaching (Denk & Svoboda 1997, Boudreau et al 2016,

Surat 2021). These effects are further exacerbated by the use of blue, energy-rich light, which is commonly used to excite green-fluorescent dyes.

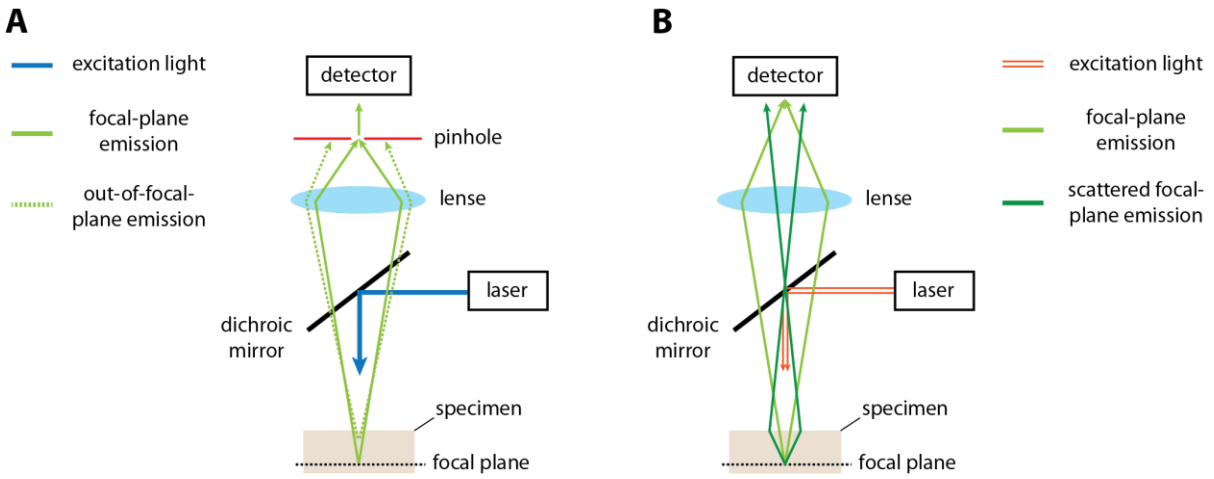

Fig 5: Schematic representation of single- and two-photon microscopy. A) Confocal microscopy: Energy-rich light excites fluorophores in the focal plane. Focal-plane emission is separated from the laser beam by a dichroic mirror. Apart from focal-plane excitation, out-of-focal-plane excitation occurs artificially causing out-of-focus emission. A pinhole filters the artificial emission; emitted light from the region of interest is passed for detection (if not scattered). **B) Two-photon microscopy:** The fusion of two lower-energy photons in the focal plane is required for excitation (red lines). This prevents the generation of out-of-focal-plane emission, which makes a pinhole obsolete. Emission light in general undergoes light scattering. However, all emitted light at a given point is attributable to the focal plane due to the two-photon principle. Therefore, scattered focal-plane emission also contributes to the signal. Adapted from Grienberger & Konnerth 2012.

Two-photon imaging has revolutionized the field of neuroscience (Denk et al 1990). Deeper brain structures up to hundreds of micrometers have become accessible (Helmchen & Denk 2005) and further improvements have even allowed imaging of small dendrites and spines in vivo in all six cortical layers (Birkner et al 2017). In TPM, two photons — each carrying about half of the energy necessary for the excitation of the fluorophore — must combine their energies. This requires very frequent and compact photon-packages (Denk et al 1990). In order to achieve this, a Ti:sapphire laser generates rapid trains of short laser pulses (Svoboda & Yasuda 2006). A high pulse frequency is crucial because two-photon consolidation only occurs within a femtosecond time window (Grienberger & Konnerth 2012). Since a single photon cannot excite a fluorophore and their consolidation is mandatory, excitation mainly occurs in the focal plane and accidental out-of-plane fluorescence is diminished. Thus, a pinhole is obsolete, because all the photons emitted in a given time — whether they are scattered or not

— are attributable to the signal (**Fig. 5 B**). Furthermore, the longer wavelength of the excitation light allows a higher penetration depth (Denk & Svoboda 1997). These characteristics lead to a higher signal-to-noise ratio and permits the imaging of deeper brain areas. As further advantages of TPM, photodamage and bleaching are reduced because lower-energy "red" photons rather than energy-rich "blue" photons are used for excitation. Light absorption and scattering of the excitation- and emission light increase with imaging depth (Centonze & White 1998, Clendenon et al 2011), therefore, the maximal imaging depth in TPM depends on the scattering factor of the tissue (Theer & Denk 2006). Scattering can nowadays be diminished by physical and chemical tissue optical clearing techniques (Yu et al 2021), however, this method is too invasive and not applicable in intact neuronal systems like acute slices or in vivo.

The maximal imaging resolution describes the shortest distance (d_{min}) at which two structures can be separated from each other. It is calculated by the quotient of the wavelength (λ) and the numerical aperture (NA), which is a dimensionless parameter of the objective and the product of the refraction index (n) of the medium between the lens and the sample and the sine of the half angular aperture of the lens (α).

$$d_{min} = \frac{\lambda}{n \cdot \sin(\alpha)}$$

The resolution increases with shorter wavelengths indicating a higher resolution in single-photon microscopy. However, in a biological sample, the image quality is more dependent on a certain imaging depth to omit surface artifacts rather than the theoretical maximal resolution. TPM permits imaging of deep, undamaged neurons in acute brain slices with sufficient resolution. Therefore, this method is appropriate to study neuronal activity by Ca^{2+} imaging. In the following, I will give a brief introduction to the fundamentals of Ca^{2+} imaging in neurons.

1.3.4 Calcium imaging in neurons

In the resting state, the cell membrane is almost uniquely permeable for potassium (Chrysafides et al 2022). The intracellular Ca^{2+} concentration at rest is about 100 nM (Berridge et al

2000), whereas the extracellular concentration is in the millimolar range (Atchison & Beierwaltes 2013). During an action potential, this gradient allows a Ca^{2+} influx through Ca^{2+}-conducting channels, resulting in a ten- to 100-fold increase of the intracellular Ca^{2+} concentration close to the membrane (Grienberger & Konnerth 2012). Dendrites are branched extensions of neurons that receive excitatory and inhibitory synaptic inputs. If an upstream excitatory neuron is activated, glutamate is released to the synaptic cleft and binds to postsynaptic AMPA and NMDA receptors within the dendritic membrane. Consequently, the channels become permeable for sodium but also for Ca^{2+} ions. The subsequent depolarization causes an excitatory postsynaptic potential (EPSP), which spreads to the neuronal soma where signals from the entire dendritic tree are integrated. Voltage-gated calcium channels are highly enriched on neuronal somata and are activated by the shifting membrane potential (Grienberger et al 2015). This results in a massive somatic Ca^{2+} influx.

The imaging of somatic Ca^{2+} levels, as used for this work, is suitable for monitoring neuronal activity levels (Mao et al 2001, Chen et al 2013). For this purpose, Ca^{2+} indicators are required to record Ca^{2+}-dependent fluorescent signals. Chemical Ca^{2+} indicators are commonly used and consist of a fluorophore and a Ca^{2+} binding site (Grienberger & Konnerth 2012). In this work, the dye Calmodulin 520 acetoxymethyl (Cal 520 AM) with an excitation wavelength of 490 nm for single- or around 920 nm for two-photon excitation and an emission peak at 520 nm is used for two-photon Ca^{2+} imaging (Tischbirek et al 2017). Since calmodulin is a physiological intracellular Ca^{2+} receptor (Means & Dedman 1980), it can be used as Ca^{2+} -binding counterpart. Due to the lipophilic character of the dye, Cal 520 AM is able to cross the membrane independent of transporters. Subsequently, the dye is processed by intracellular esterases that split lipophilic groups off the indicator. Hereby, the remaining fluorescent molecule is captured intracellularly because it becomes electrically charged and more hydrophilic. It is possible to stain a large neuron population at once by applying a bolus of Cal 520 AM in a respective area. For this work, a dye-containing glass pipette is navigated to the CA1 area of the hippocampus and the indicator is pressure-applied into the extracellular space. The lipophilic non-cleaved molecules are washed out from the tissue, resulting in a high signal-to-noise ratio (**Fig. 6**).

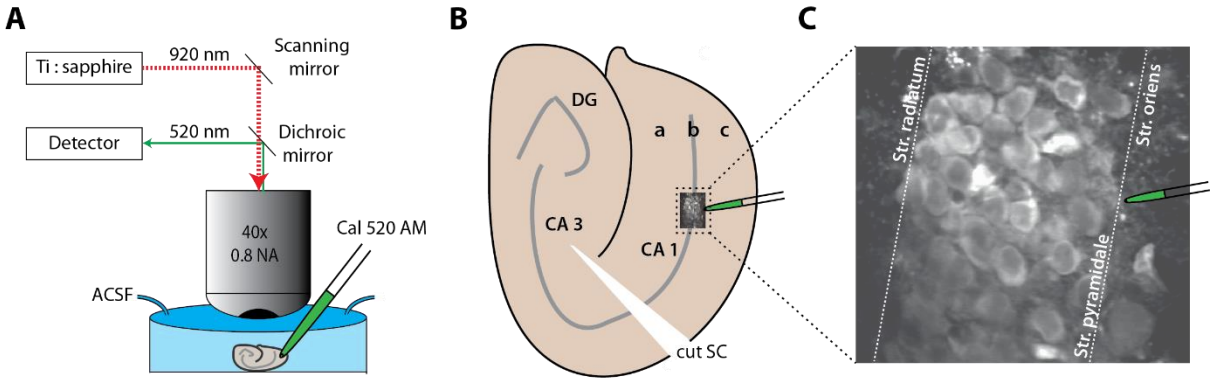

Fig. 6: Multicell bolus loading of Cal 520 AM to stratum pyramidal neurons in the hippocampal CA1 area. A) The brain slice is superfused with ACSF in a two-photon microscope recording setup. A pulsed Ti:sapphire laser at a wavelength of 920 nm is used for excitation and emitted photons with a wavelength of 520 nm are detected by a hybrid photomultiplier tube. **B)** Pressure application of the Ca^{2+} indicator from the stratum oriens (c) to neurons in the stratum pyramidale (b). Stratum radiatum (a). **C)** Representative two-photon image from the area described in B) after 30 minutes of incubation.

It was shown that (i) Cal 520 AM is sufficient to detect single action potentials, (ii) the amplitude of the signal correlates with the number of spiking trains at 20 Hz, and (iii) in a train of five, single spikes can be separated as single Ca^{2+} peaks at a frequency of 10 Hz or less (Tada et al 2014). Overall, these properties indicate that Cal 520 AM is an appropriate indicator to quantify neuronal activity in acute preparations. Even though AM indicators can be used after a prolonged incubation (Cameron et al 2016), they are basically washed out from the tissue, making them unfeasible for long-term imaging studies. An alternative approach is the expression of a genetically encoded calcium indicators (GECIs) (Grienberger & Konnerth 2012). In contrast to acute bolus loading, GECIs can be expressed in specific cell types and the method allows long-term imaging in vivo (Zhong & Schleifenbaum 2019). However, this approach is time-consuming, more complex, and the advantages are not required for this work.

In summary, multicell bolus loading of Cal 520 AM is a reliable and reproducible method to permit Ca^{2+} imaging in large neuron populations. Since the somatic Ca^{2+} concentration reflects the activity level of a neuron, this approach allows the investigation of how Aβ peptides affect the activity level of pyramidal neurons in vitro.

2 Methods

2.1 Slice preparation

All experiments were conducted in accordance with animal welfare standards. Wild-type C57BL/6 mice were bred in the local animal house. Because tissue viability decreases with age (Staal et al 2011), I used 9 to 16 postnatal day-old mice of both sexes in line with general practice in the field (Stosiek et al 2003). Mice were anesthetized in a small chamber with pure CO_2 and immediately decapitated with a guillotine. The brain was surgically removed (**Fig. 7 A**) and quickly transferred into ice-cold cutting solution (2.5 mM KCl, 26 mM $NaHCO_3$, 65.5 mM NaCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 24.7 mM glucose, 105 mM sucrose, and 1.7 mM ascorbic acid with an osmolarity of 330 mOsm) (Lein et al 2011). The pH value was adjusted to 7.4 and stabilized by bubbling with carbogen gas (Vol. 5 % CO_2, Vol. 95 % O_2). Then, the brain was glued onto a plate and fixed in the chamber of a vibratome (VT1200S; Leica, Germany). The chamber was filled with ice-cold cutting solution and the brain was covered during slicing (**Fig. 7 B**). Horizontal 300 µm thick slices were prepared and hippocampal slices were put into the recovering solution (2.5 mM KCl, 26 mM $NaHCO_3$, 119 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 12.5 mM glucose, 3 mM Na-pyruvate (Sigma), 5 mM Na-ascorbate (Sigma), 2 mM thiourea (Sigma), and 1 mM glutathione monoethyl ester). The osmolarity was 290 mOsm, the pH value was adjusted to 7.4 with HCl and stabilized by bubbling with carbogen gas at room temperature (**Fig. 7 C**). After a recovery period of at least 30 minutes, a slice was transferred into a custom-made chamber and fixed with a grid consisting of fine filaments glued onto a small platinum frame. Using a common light microscope, Schaffer collaterals (SCs) were cut with a scalpel to prevent epileptiform spontaneous baseline activity (**Fig. 7 D**). Then, the chamber was fixed in the TPM setup and the acute slice was superfused with heated (37 °C) ACSF during the entire experiment (4.5 mM KCl, 26 mM $NaHCO_3$, 125 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 20 mM glucose, pH value of 7.4 stabilized with carbogen).

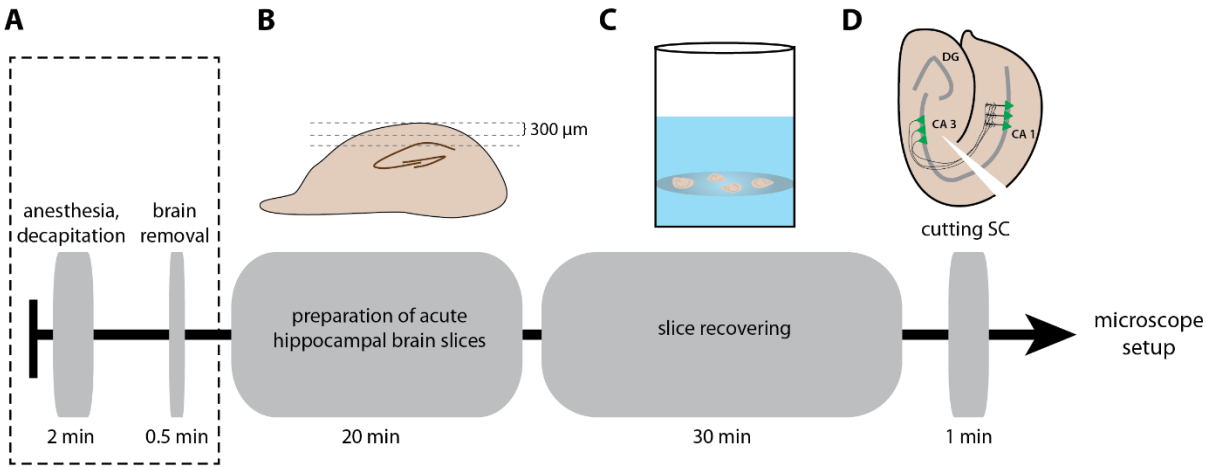

Fig. 7: Schema of acute hippocampal mouse slice preparation. A) One animal is anesthetized and decapitated. The brain is quickly removed and transferred to an ice-cold cutting solution. **B)** The brain is fixed horizontally and cut into 300 µm thick slices. **C**) Only slices containing the hippocampus are placed in a chamber for recovery. **D**) Schaffer collaterals (SCs) are cut before transferring the tissue to the microscope setup.

2.2 Staining

The Ca^{2+} indicator Cal 520 AM (AAT Bioquest: 50 µg) was dissolved in 4 µl Pluronic (Invitrogen: Vol. 20 % Pluronic, Vol. 80 % DMSO) and mixed for two minutes. Alexa Fluor 488 (Invitrogen, Thermo Fisher) was diluted in Ca^{2+} -free ringer solution (2.5 mM KCl, 150 mM NaCl, 10 mM HEPES, osmolarity: 300 mOsm, pH value: 7.4) to a concentration of 10 µM. Then, 76 µl of the solution was added to the Ca^{2+} indicator to achieve a final Cal 520 AM concentration of 0.5 – 1.0 mM. For the application of the dye and Aβ preparations, glass micropipettes were produced with a puller (Narishige, PC-10). Each pipette tip underwent a quality control with a light microscope and had a tip resistance of 1 - 3 MΩ. For staining, a micropipette was filled with 3 µl of the solved dye and fixed in a micromanipulator (Luigs & Neumann, Germany). The two-photon setup was equipped with a light source that facilitated the navigation of the pipette within the ACSF solution. The hippocampal formation was identified morphologically and the micropipette was lowered to the surface of the hippocampal CA1 area. The pipette was gently inserted from the stratum oriens site (**Fig. 6 B, C**) and pushed forward to a depth of about 150 µm into the CA1 stratum pyramidale. The tip was located by exciting Alexa Fluor 488 with the laser because Cal 520 AM is non-fluorescent in Ca^{2+} -free solution in the pipette. For multicell bolus loading, I carefully applied increasing pressure (PDES-01AM,

NPI Electronic, Germany) to the pipette in order to inject the indicator into the extracellular space (Garaschuk & Konnerth 2010, Stosiek et al 2003). Simultaneously, the area around the pipette tip was imaged. As soon as an Alexa 488 signal became visible as an increase of fluorescence outside the pipette, the pressure (≈ 200 mbar) was kept constant for 30 seconds. Aβ application experiments were started after an incubation period of at least 30 minutes.

2.3 Two-photon calcium imaging

I used a custom-build upright TPM setup (Olympus) and a tunable Ti:sapphire laser (Mai Tai® DeepSee™, Spectra-Physics) at a wavelength of 920 nm, a pulse-width of 75 fs, and a frequency of 80 MHz. The laser power was adjusted with a pockels cell (Conoptics) to values of 20 – 40 mW. A scanning-unit guided the laser beam rectangularly over the sample. This was achieved by using a galvanometric resonant mirror scanning system (line rate: 12 kHz). The setup was equipped with a water immersion objective (Nikon, 40x, NA: 0.8). Custom-written software based on the Laboratory Virtual Instrument Engineering Workbench (LabVIEW, National Instruments) enabled manipulation of the microscope and data acquisition. One field of view (FOV) consisted of 600 x 600 pixels (≈ 80 x 80 µm) and was scanned with an image frame rate of 40 Hz. Emitted photons were separated from the laser beam by a dichroic mirror and the signal was amplified and detected with a hybrid photomultiplier tube (Hamamatsu). Gathered data was stored locally for further offline analysis.

2.4 Activity induction

Neurons in acute hippocampal brain slices have no significant spontaneous baseline activity under physiological conditions (**Fig. 3**). Since CA1 pyramidal neurons receive both excitatory and inhibitory synaptic inputs (Megías et al 2001), a single increase of the potassium concentration would unpredictably affect the E/I ratio. Therefore, I added 100 µM of the competitive $GABA_A$ – receptor antagonist bicuculline (Enzo Life Sciences) to the ACSF solution for synaptic disinhibition and tightly increased the extracellular potassium concentration to 5.5 – 7.5 mM for synaptic excitation (**Fig. 8 A, B**). Both agents generated in vivo-like activity on their own. However, their combination yielded a more stable baseline over longer periods of time. Glutamate is also capable of inducing activity (Zott et al 2019) (**Fig. 8 C**), even though it was not

used in this work. SCs were cut to prevent the formation of excessive synchronized baseline activity (**Fig. 7 D**) because a raised potassium concentration is known to be epileptogenic (Liu et al 2021).

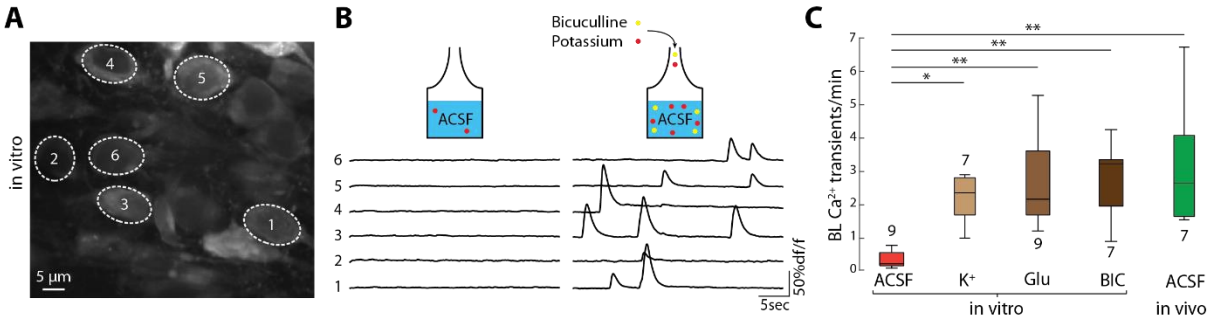

Fig. 8: Induction of in vivo-like spontaneous baseline activity in vitro. A) In vitro two-photon image of CA1 pyramidal neurons. **B)** Baseline Ca^{2+} transients of six neurons circled in A) before (left) and after (right) adding bicuculline and additional potassium to the ACSF solution. **C)** Box plots of mean baseline activity levels under different in vitro conditions compared to the in vivo situation. The number of slices (in vitro) and animals (in vivo) are marked for each assay. Kruskal Wallis test with Dunn-Sidak post-hoc comparison. * $p < 0.05$, ** $p < 0.005$. (C) was adapted from Zott et al 2019 with permission from AAAS.

2.5 Synthetic Aβ preparations

Monomers

Synthetic Aβ 1-40 monomers were purchased (Bachem), dissolved in DMSO (Sigma-Aldrich), and diluted to a concentration of 5 mM. Small volumes were filled into low protein binding tubes (Eppendorf) and stored at - 20 °C to stabilize their monomeric state. Since the dilution of monomers in aqueous solution triggers the aggregation process (Stine et al 2003), one single aliquot was defrosted and diluted in ACSF with adjusted amounts of bicuculline (100 µM) and potassium (5.5 – 7.5 mM) to an Aβ concentration of 1 or 100 µM immediately before the experiment to keep the defrost-to-application-time as short as possible.

Putative oligomers

I developed an assay to produce Aβ aggregates by incubating dissolved Aβ 1-40 monomers. A stored low protein binding tube containing 5 mM of synthetic Aβ 1-40 monomers was defrosted and Aβ was diluted in ACSF to 100 µM (Rauth et al 2016). For oligomerization, the solution was incubated for 0.5 – 2.5 h at room temperature in the dark without shaking. Then,

the tube was centrifuged (2000 x g, 6000 min^{-1}) for three minutes. The supernatant was applied to neurons in a concentration of 1 µM (diluted) or 100 µM.

2.6 Human Aβ preparations

I used purified human-derived Aβ species that were provided by the Dominic Walsh laboratory. Human brain extract was obtained from patients who died with a diagnosis of AD. They showed impaired cognitive performance as measured by Mini-Mental State Examination and their brains revealed AD-specific alterations. The preparation of aqueous brain extract was described previously (Wang et al 2017). Briefly, gray matter was dissected from the white matter and cortical tissue was dissolved in buffer (124 mM NaCl, 2.8 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, pH 7.4) supplemented with 5 mM EDTA, 1 mM EGTA, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml pepstatin, 120 µg/ml pefabloc and 5 mM NaF for homogenisation. After centrifugation for 110 minutes at 4 °C, the upper 90 % of the supernatant was dialysed against fresh buffer solution. A portion of the extract passed through several rounds of incubation with the anti-Aβ antibody AW7 plus Protein A Sepharose to remove Aβ in order to obtain Aβ-free immunodepleted brain extract (**ID-ex**). The other part was mock immunodepleted (**AD-ex**). Both samples were stored at - 80 °C. It is not possible to calculate the exact Aβ concentration of the solution as it contains a variety of different Aβ molecules of unknown size. Respective aliquots were thawed immediately before the experiment, diluted 1:60 in ACSF with adjusted amounts of bicuculline and potassium, and applied to neurons.

The extraction of human Aβ monomers and dimers from the brain extract and Aβ plaques was also previously described. The procedure involves a complex sequence of incubation, homogenisation, boiling in water, centrifugation and washing steps, as well as filtration through nylon meshes with decreasing permeability (for a detailed description see Brinkmalm et al 2019). The solution underwent congo red staining, polarizing microscope visualisation, western blot analysis, and size-exclusion chromatography for characterization. Aβ fractions of 7 kDa were collected and identified as human Aβ dimers and Aβ fractions of 4 kDa were recognized as human Aβ monomers. According to the molecular weights, peptide concentrations were estimated to be in the nanomolar range (Zott 2019). Small portions of both fractions were filled into tubes and stored at - 80 °C at a concentration of 1 µg/ml. For experiments,

the extracts were diluted 1:5 or 1:20 in ACSF adjusted for bicuculline and potassium and applied locally. Once defrosted, the respective aliquot was discarded after the experiment.

2.7 Aβ application

2-5 µl of the respective Aβ-containing solution or vehicle were filled in a micropipette and the tip was carefully navigated close to pyramidal neurons under visual control via the microscope. After recording the spontaneous neuronal baseline activity for 60 seconds, the solution was gently pressure-applied (≈ 150 mbar) to neurons for 30 seconds under visual control. It is important to apply the solution carefully to reduce mechanical artifacts. After a washout of three minutes, spontaneous activity was recorded again for a period of 60 seconds.

2.8 Data analysis

Raw data was imported into a custom-written software based on LabView (Jia et al 2011). Neuronal somata were visually identified and marked as ROIs. Only somata that were trackable during the entire application were included because occasionally, the pressure application of Aβ pushed individual somata out of the FOV. Relative fluorescence Δf(t) was calculated as change of fluorescence f(t) - f_0 per time point t normalized to the baseline fluorescence f_0 for each ROI for baseline, Aβ application and washout.

$$\Delta f(t) = \frac{f(t) - f_0}{f_0}$$

Ca^{2+} traces were stored and imported to Igor Pro (Wavemetrics, USA) to count the Ca^{2+} transients for each experiment. Peaks were counted as transients if their amplitudes were at least two times larger than the width of the noise band. Furthermore, neurons with a baseline frequency of more than 20 transients / min were excluded from the analysis.

The distance of neurons to the application site was classified categorically. Pipettes were constantly navigated to the left-middle site (perspective from the TPM image) of the FOV. The area located directly around the tip was marked as zone A (least distance); cells placed close to the margin of the FOV were allocated to zone C (largest distance). All remaining cells were

included in zone B between A and C. Change of neuronal activity $\Delta_{activity}$ was calculated as the difference between the activity level during the application (Ca^{2+} peaks $_{Appl}$) and under baseline conditions (Ca^{2+} peaks $_{BL}$) per minute.

$$\Delta_{activity} = \left(Ca^{2+}\ peaks_{Appl(30s)} \times 2\right) - \left(Ca^{2+}\ peaks_{BL(60s)}\right)$$

2.9 Statistics

Statistical analysis was executed in Python using the module scipy.stats in Jupyter Notebook. Two-sided wilcoxon signed-rank tests (Wilcoxon test) and Wilcoxon rank-sum tests (Mann-Whitney U test, MWU) were commonly used unless otherwise specified. The significance level was set to p-values < 0.05 ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Errors of means were denoted as standard error of the mean (SEM).

3 Results

For my thesis, I used two-photon Ca^{2+} imaging in vitro to investigate the hyperactivity-inducing potential of different Aβ aggregates on a single cell level. TPM was used because — apart from a high spatiotemporal resolution — a sufficient penetration depth is crucial to omit the imaging of slicing-related tissue damage. Furthermore, somatic Ca^{2+} imaging is suitable for detecting Aβ-mediated changes in neuronal activity (Mao et al 2001) as an action potential triggers an increase of the intracellular Ca^{2+} concentration (Grienberger & Konnerth 2012). Since a systematic analysis of the hyperactivity-inducing potential of different Aβ species such as monomers, dimers, and oligomers is still lacking and some evidence have already indicated that Aβ monomers and dimers differ in toxicity (Shankar et al 2007), it is crucial to examine whether and how the size (and source) of Aβ affect the toxicity of a respective peptide. Therefore, the aim of this work is to investigate whether (i) synthetic/human-derived Aβ monomer/dimers, (ii) Aβ-containing brain extract from AD patients, and (iii) Aβ aggregates formed by oligomerization of synthetic Aβ 1-40 monomer have the ability to induce neuronal dysfunction. All in vitro experiments in this work were conducted under equivalent conditions with cut SCs as well as bicuculline- and potassium-induced spontaneous baseline activity in the hippocampal CA1 area in acute brain slices from wild-type mice. All slices were stained with the Ca^{2+} indicator Cal 520 AM. Once the activity increased to stable in vivo-like levels, the baseline was recorded before different forms of Aβ were gently applied to pyramidal neurons. During the application and after washout, neuronal Ca^{2+} transients were recorded to quantify the neurotoxic potential of the applied Aβ.

3.1 Synthetic Aβ monomers and Aβ aggregates vary in hyperactivity-inducing potential

In previous experiments in our laboratory, synthetic Aβ dimers had initially no effect on silent acute slices (**Fig. 3 B, D**). However, they potently induced neuronal hyperactivity in vitro (**Fig. 4**) after the spontaneous baseline activity was raised to in vivo levels (**Fig. 8**). As a first step, I tested the effect of synthetic Aβ monomers.

3.1.1 Synthetic Aβ monomers are not neurotoxic

Initially, I asked whether Aβ monomers also induce neuronal hyperactivity in vitro. This is a fundamental question because there is evidence that monomers and dimers differ regarding their neurotoxic potential (Walsh et al 2002, Klyubin et al 2005). Since dimers may be formed by the assembly of monomers, investigating their neurotoxicity appears even more important. Although monomers have been already tested in several assays, there is — at least to my knowledge — no evidence of their hyperactivity-inducing potential.

Application of highly diluted Aβ 1-40 monomers

In the first experiment, I tested the effect of synthetic Aβ monomers in a concentration of 1 µM, which is the same as in the Aβ dimer experiment. As soon as neurons revealed a stable in vivo-like baseline activity, I filled a pipette with synthetic Aβ 1-40 monomers. The pipette was carefully navigated to the pyramidal cells to a depth of at least 50 µm to avoid the imaging of superficial slicing artifacts. After recording the baseline activity, the solution was applied to the neurons and the activity was recorded again after washout. The application of 1 µM Aβ 1-40 monomers had no effect on the baseline activity of the selected neurons (**Fig. 9**).

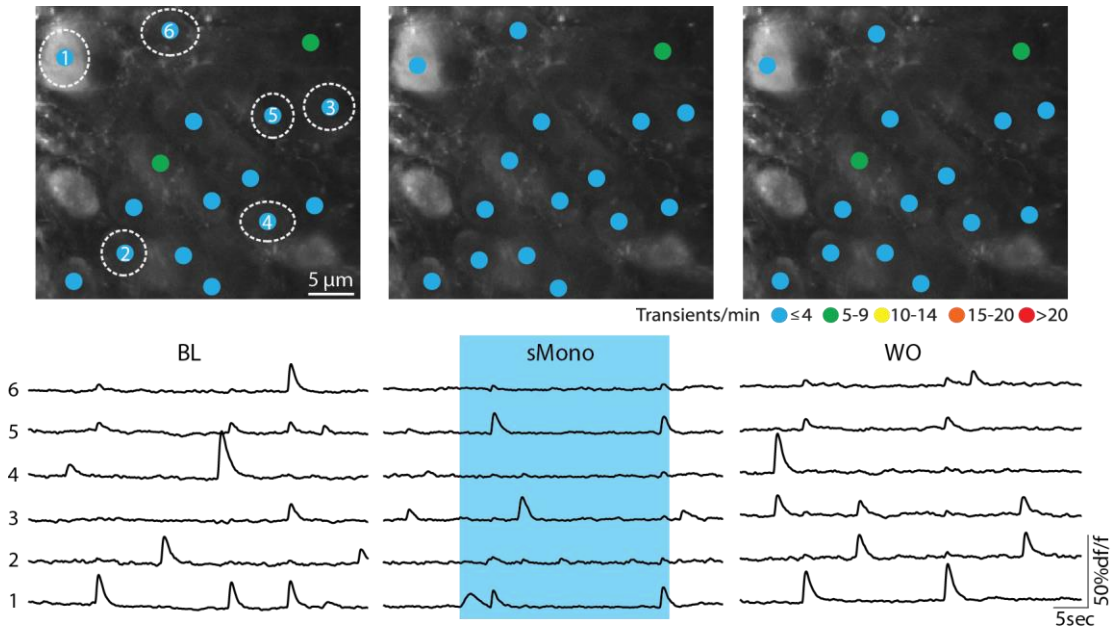

Fig. 9: Synthetic Aβ 1-40 monomers do not induce neuronal hyperactivity. Two-photon images (up) with five circled cells with corresponding Ca^{2+} traces (down) for baseline, Aβ 1-40 monomer application (1 µM, shaded area) and washout. Colored dots represent the number of Ca^{2+} transients per minute during each phase.

Application of highly concentrated Aβ 1-40 monomers

Next, in order to exclude that the initial concentration of Aβ monomers had been too low, the Aβ 1-40 monomer concentration was raised to 100 µM. Although increasing monomer concentrations are in general more prone to aggregate, one study has suggested that the respective peptide concentration is initially monomeric in solution (Goldsbury et al 2000). Similar to the 1 µM application, the application of 100 µM monomers did not induce neuronal hyperactivity (**Fig. 10**).

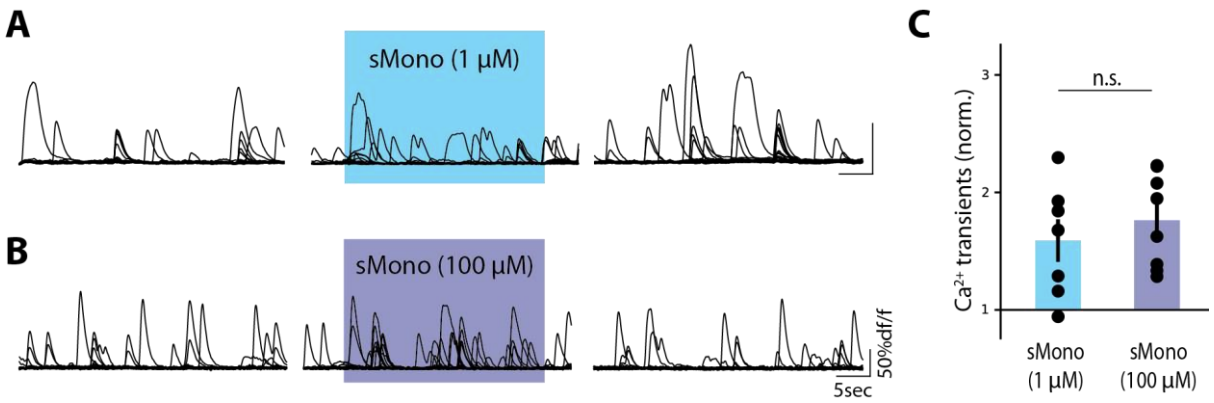

Fig. 10. Synthetic Aβ 1-40 monomers even at high concentrations do not induce neuronal hyperactivity. A) Overlayed Ca^{2+} traces of cells (z = 28) of one experiment from Fig. 9 for baseline (left), 1 µM monomer application (shaded area, middle) and washout (right). **B)** Same as A) for 100 µM (z = 24). **C)** Bar graph of mean Ca^{2+} transients normalized to baseline for 1 µM (n = 7, left) and 100 µM (n = 8, right) synthetic Aβ monomers. Error bars display SEM, MWU test (C). n.s. not significant.

3.1.2 Putative Aβ 1-40 oligomers induce neuronal hyperactivity

My previous experiments are in line with a review suggesting that Aβ monomers are not neurotoxic (Qiu et al 2015). It has also been reported that Aβ toxicity increases in a rank order from monomers to larger oligomers (Hayden & Teplow 2013), however, very large Aβ aggregates such as fibrils and plaques have no effect on learning, memory, and synaptic plasticity, in contrast to AβOs (Verma et al 2015, Shankar et al 2008). Since Aβ dimers induce neuronal hyperactivity, it is reasonable to assume that the damaging potential arises early within the aggregation cascade. AβOs have been identified as early Aβ aggregates formed by assembling

monomers (Shankar et al 2008), and notably, it was already shown that the aggregation of initially innocent Aβ 1-40 monomers yields aggregates that affect the electrophysiology in cortical neurons (Hartley et al 1999). However, whether Aβ monomers can also acquire hyperactivity-inducing potential remained unknown. In order to address this question, I developed an aggregation assay to generate Aβ aggregates out of Aβ monomers. The aggregation is mainly determined by the initial peptide concentration, among other factors. One study showed that Aβ 1-40 aggregation needs a monomer concentration of greater than 14 µM (Sengupta et al 2003); similar data revealed that Aβ 1-40 fibrillization requires an Aβ concentration of 10 - 40 µM (Finder & Glockshuber 2007). Furthermore, it was reported that after two days of incubation, 75 µM of Aβ 1-40 monomers form stable dimers (Garzon-Rodriguez et al 1997). Finally, Sengupta and colleagues demonstrated a remarkable aggregation of 158 µM Aβ 1-40 after only 45 to 60 minutes (Sengupta et al 2003). Together, even though these results cannot be properly compared, there is a tendency confirming that a larger peptide concentration needs less time to form Aβ assemblies, e.g., 231 µM might only need one to two hours (Rauth et al 2016).

Formation of Aβ aggregates

The assembly of Aβ 1-40 monomers has also been systematically studied by Goldsbury and colleagues in vitro. They showed that the incubation of 100 µM of Aβ 1-40 monomers results in a rapid increase of soluble AβOs and a simultaneous decrease of Aβ monomers (Goldsbury et al 2000). Respectively, I diluted synthetic Aβ 1-40 monomers in ACSF to 100 µM in order to collect small Aβ aggregates in accordance with this data. After an incubation time of 0.5 – 2.5 h, 2-5 µl were filled in a pipette for local application. However, the pipette became blocked immediately after pressure injection, and it remained impossible to apply the solution even after increasing the pressure extensively. Puzzled, I removed the pipette and examined the tip under the light microscope. I remarked that visible sludge had clogged the pipette tip and concluded that the incubation had yielded high-molecular weight Aβ aggregates. Therefore, I decided to centrifuge the solution for three minutes after incubation to separate smaller from larger Aβ aggregates. After filling a micropipette with a few microliters of the supernatant, the solution could be gently reapplied to the pyramidal neurons (**Fig. 11**).

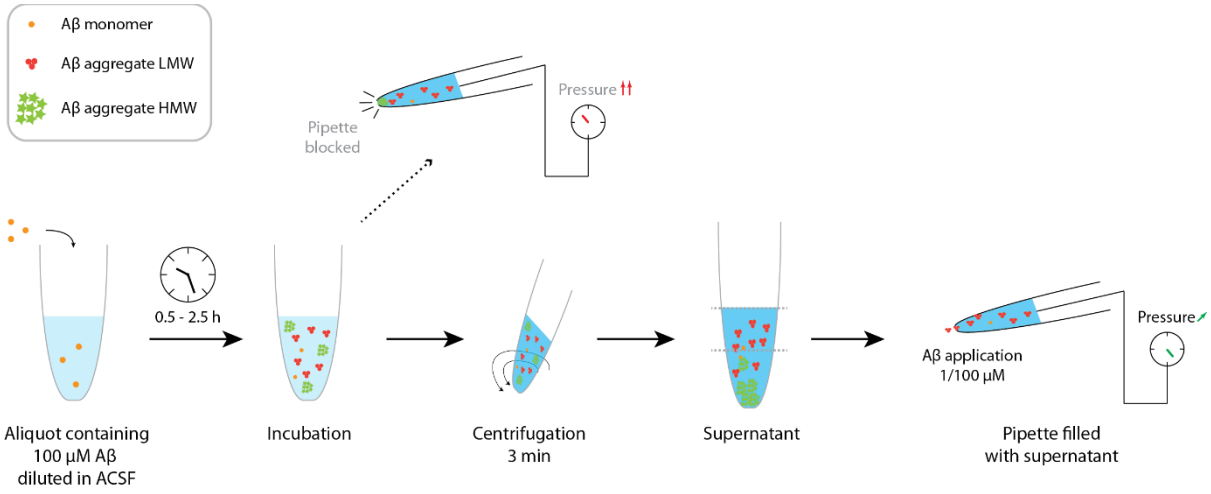

Fig. 11: Formation of Aβ aggregates. Synthetic Aβ 1-40 monomers were diluted in ACSF to a concentration of 100 µM and incubated for 0.5 – 2.5 h in the dark at room temperature. After three minutes of centrifugation, some microliters of the supernatant were filled in a glass pipette for local application. Without centrifugation, visible sludge, likely HMW Aβ aggregates, blocked the pipette tip and prohibited application. LMW: Low-molecular-weight, HMW: High-molecular-weight

Application of highly concentrated Aβ aggregates

Since the application of incubated Aβ monomers was impossible without centrifugation and a cooperating laboratory obtained a significant amount of thioflavin-positive amyloid aggregates within an hour using a similar protocol (Rauth et al 2016), I assumed that my aggregation protocol is, in principle, sufficient to form Aβ assemblies (**Fig. 11**). In order to test whether Aβ monomers acquire hyperactivity-inducing potential, I similarly applied 100 µM of aggregated Aβ to pyramidal neurons. Remarkably, the application induced neuronal hyperactivity in almost all neurons in the FOV (**Fig. 12 A**) and despite the immediate and strong effect, the neuronal activity returned to baseline levels after washout (**Fig. 12 B**).

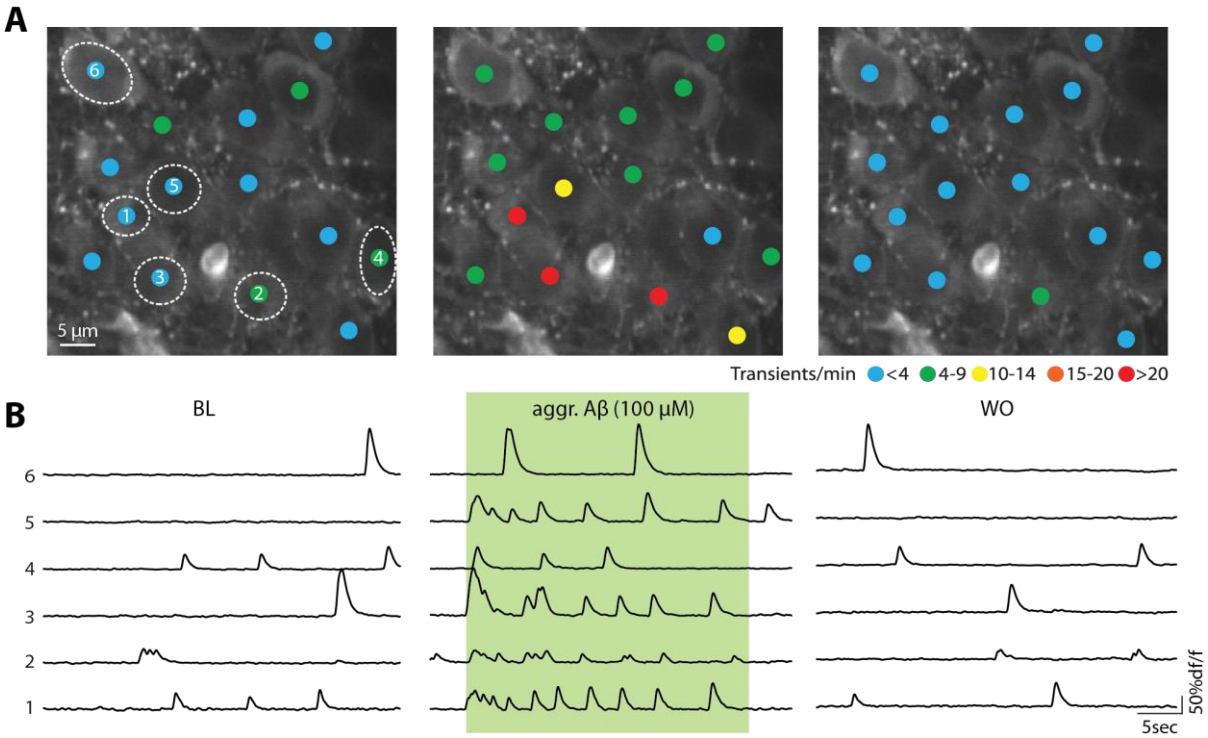

Fig. 12: Aggregated Aβ 1-40 monomers potently induce hyperactivity. A) Representative two-photon image with colored dots representing the number of Ca^{2+} transients per minute during baseline, Aβ aggregates (100 µM) application and washout (from left to right). **B)** Ca^{2+} traces of six selected cells circled in A) for baseline, application (shaded area) and washout.

The application caused a reliable increase of neuronal activity in all experiments conducted (**Fig. 13 A**). Comparing the effect of Aβ monomers before and after aggregation, aggregated Aβ strongly induced neuronal hyperactivity while freshly dissolved Aβ monomers were ineffective (**Fig. 13 B**). Within this context, it is important to mention that the final concentration of aggregates was adopted from the initial Aβ monomer concentration. Naturally, the concentration of Aβ aggregates in this solution was lower than 100 µM and depended on the size of the respective molecules.

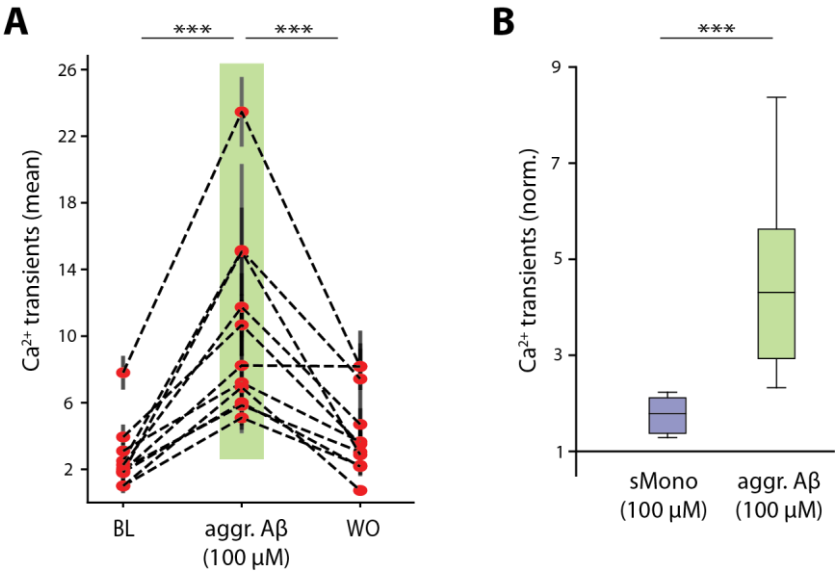

Fig. 13: Aggregated Aβ 1-40 monomers obtain hyperactivity-inducing potential. A) Quantitative data for the experiment shown in Fig. 12. Dots indicate the mean of Ca^{2+} transients per minute for baseline, aggregated Aβ (100 µM) and washout. Lines connect dots of one single experiment (n = 11). **B)** Box plot of mean Ca^{2+} transients during application normalized to baseline for unaggregated (n = 8, left) and aggregated (n = 11, right) 100 µM Aβ monomers. Error bars display SEM. Wilcoxon test (A), MWU test (B). *** $p < 0.001$.

Application of highly diluted Aβ aggregates

In the previous experiment, I showed that aggregated Aβ monomers (100 µM monomer equivalent) potently induced neuronal hyperactivity. However, this high concentration does not accurately represent the in vivo situation. The estimated concentration of Aβ in healthy brains is in the picomolar range (Lazarevic et al 2017) and is likely to increase further in AD, however, it will not reach large micromolar values. In order to consider this issue at least in part, I tested whether 1 µM of Aβ aggregates remains neurotoxic. Remarkably, highly diluted Aβ aggregates also induced neuronal hyperactivity (**Fig. 14**).

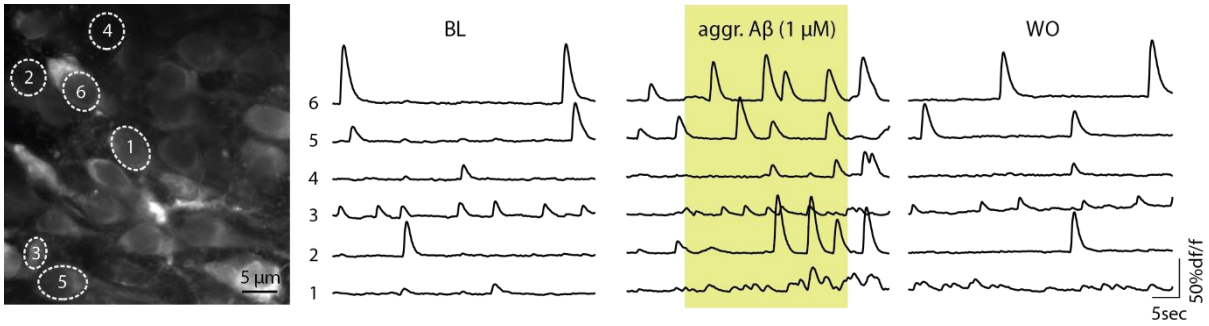

Fig. 14: Aβ aggregates induce neuronal hyperactivity even at a low concentration. Representative two-photon image with six circled cells and corresponding Ca^{2+} transients for baseline, application of Aβ aggregates (1 µM, shaded area) and washout (from left to right).

Similar to the last preparation, the quantification revealed a significant increase of the activity from baseline to application (**Fig. 15 A**)**.** Moreover, the effect remained statistically different from freshly dissolved highly diluted Aβ monomers (**Fig. 15 B**). Remarkably, the hyperactivating potential of the aggregates increased with longer aggregation times (**Fig. 15 C**), which indicates that either neurotoxic species became available at higher concentrations or that the aggregates generated had a higher toxic potential.

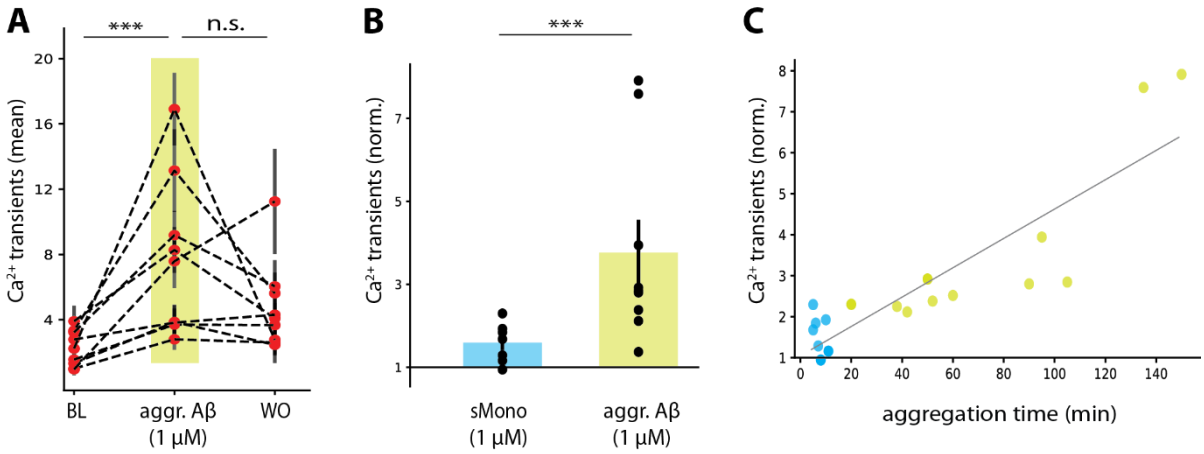

Fig. 15: Neurotoxicity of Aβ aggregates is preserved even at low concentrations. A) Quantitative data for the experiment shown in Fig. 14. Dots indicate the mean of Ca^{2+} transients per minute for baseline, aggregated Aβ (1 µM) and washout. Lines connect dots of one single experiment (n = 9). **B)** Bar graph of mean Ca^{2+} transients normalized to baseline for unaggregated (n = 7, left) and aggregated (n = 9, right) 1 µM Aβ monomers. **C)** Scatter plot of the data shown in B) as a function of the aggregation time. The line represents the trendline. Error bars display SEM. Wilcoxon test (A), MWU test (B). *** $p < 0.001$, n.s. not significant.

3.1.3 Neuronal hyperactivation is attributable to Aβ

As a control experiment, I inquired whether neuronal hyperactivation could also be caused by local vehicle application, for instance, through mechanical manipulation itself. In order to test this, ACSF was applied to CA1 pyramidal neurons equivalent to the Aβ solutions. In this context it is important to mention that the same ACSF solution superfusing the slice, adjusted for bicuculline and potassium, was used for all experiments because deviating potassium and bicuculline concentrations may alter neuronal activity levels independently of Aβ. As expected, the vehicle application did not affect neuronal activity levels (**Fig. 16 A, B**). The effects of synthetic Aβ aggregates differed significantly from those of ACSF application, and there was no difference between the control experiment and synthetic monomer application. Therefore, I concluded that monomers indeed do not carry hyperactivity-inducing potential (**Fig. 16 C**).

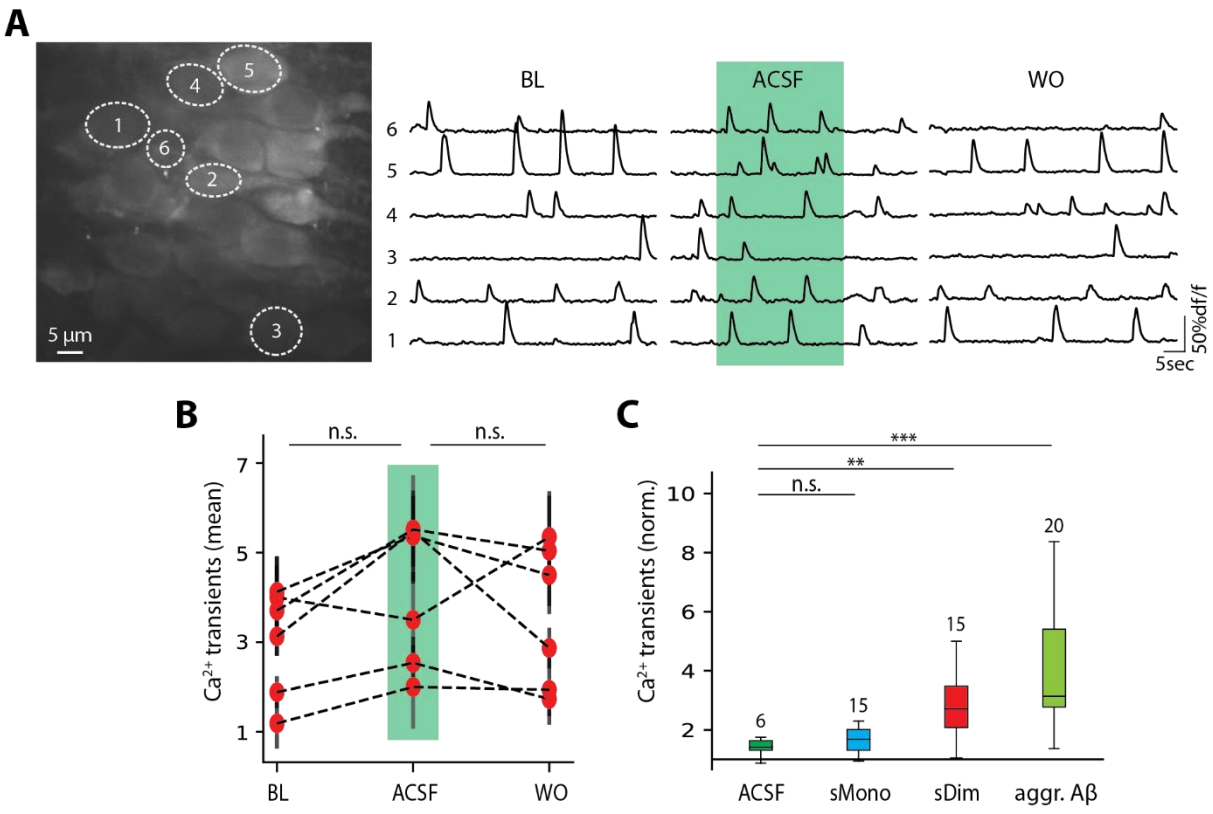

Fig. 16: Vehicle application does not alter the neuronal activity level. A) Representative two-photon image with six circled cells and corresponding Ca^{2+} transients for baseline, ACSF application (shaded area) and washout (from left to right). **B)** Quantification of data shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one coherent experiment (n = 6). **C)** Box plots of mean Ca^{2+} transients normalized to baseline for experiments with synthetic Aβ peptides. Total numbers of experiments are indicated over the respective plots. Error bars display SEM. Wilcoxon test (B), MWU test (C). ** $p < 0.01$, *** $p < 0.001$, n.s. not significant.

Our group previously discovered a positive correlation between the baseline activity level and the extent of hyperactivation (Zott et al 2019). Therefore, a low baseline activity in control experiments could mask an activating effect of the vehicle. Thus, I next analyzed the baseline activity for each experiment shown in Fig. 16 C on a single cell level (**Fig. 17 A**). The spontaneous baseline activities of all experiments were similar and did not vary significantly (**Fig. 17 B**), excluding differences in baseline activity as a possible confounding factor.

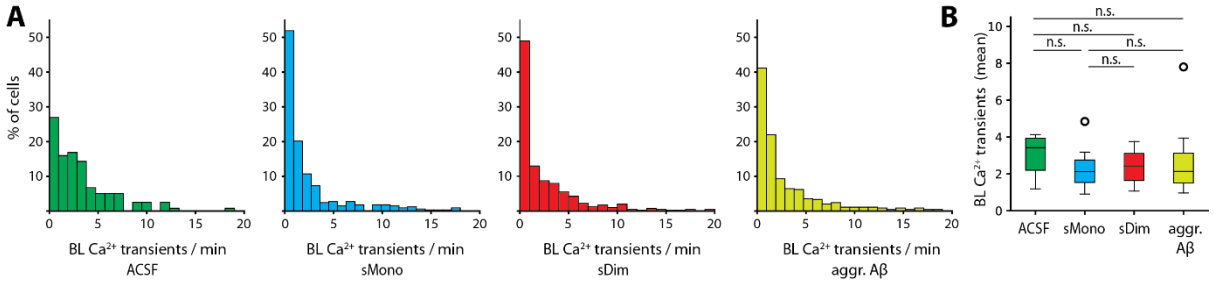

Fig. 17: Baseline activity levels do not differ within synthetic Aβ application experiments and vehicle control. A) Histogram of Ca^{2+} transients per minute during baseline for ACSF (n = 6), synthetic Aβ monomers (n = 15), Aβ dimers (n = 15) and Aβ aggregates (n = 20) applications (from left to right). **B)** Box plots of data shown in A). MWU test (B). n.s. not significant.

Lastly, a quantitative single-cell analysis in vitro revealed that the hyperactivating effect of Aβ is pronounced in cells located close to the application site (**Fig. 18 A, B**). This might indicate that the local manipulation itself affects the baseline activity instead of Aβ peptides. However, comparable results could not be achieved with the application of ACSF solution (**Fig. 18 C**). Therefore, the observed gradient is likely caused by a concentration gradient of Aβ with increased Aβ peptide concentrations close to the pipette tip which decreases with growing distance.

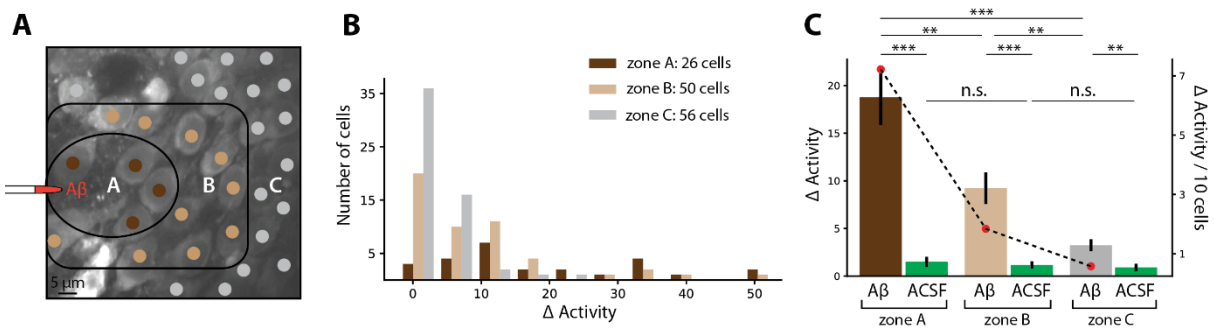

Fig. 18: Neuronal hyperactivation depends on Aβ and decreases with growing pipette distance. A) Classification schema of neurons located close to the pipette (zone A), in the outer part of the FOV (zone C) or in between (zone B). **B)** Histogram showing the gain of activity from baseline to Aβ application (Δ Activity) for human AD brain extract experiments (n = 6, z = 132, see chapter 3.2.1) separated for zones shown in A). **C)** Bar graph of means of data shown in B) and ACSF control experiments (green bars, n = 6, z = 125) for respective zones. Red dots indicate the normalized activity change per ten cells. Error bars display SEM. MWU test (C). ** $p < 0.01$, *** $p < 0.001$, n.s. not significant.

3.2 Human-derived Aβ causes neuronal hyperactivity

For the next series of experiments, our laboratory worked together with a cooperating research group. They developed a protocol to isolate Aβ-containing extract from human AD brain samples (Hong et al 2018). Furthermore, they extracted and purified human Aβ monomers and dimers from the respective extracts (Brinkmalm et al 2019). While the majority of studies used synthetically produced Aβ peptides, growing evidence denoted a greater potency of these human-derived Aβ species (Li & Selkoe 2020). Notably, human Aβ peptides physiologically undergo post-translational modifications that alter their neurotoxic potential (Barykin et al 2017). Within this context, it is interesting to equally study their hyperactivity-inducing potential.

3.2.1 Brain extract from AD patients induces activity-dependent neuronal hyperactivity

Initially, I tested a human-derived brain extract (**AD-ex**) that contains a large heterogeneity of Aβ. This approach mimics the in vivo situation since different Aβ species coexist simultaneously (Selkoe & Hardy 2016). As the exact composition of AD-ex is unknown, it is impossible to determine the concentration of Aβ (Zott 2019), but it can be estimated in the nanomolar range. Notably, AD-ex contains far more Aβ aggregates than monomers (Zott et al 2019).

AD-ex induces neuronal hyperactivation

In order to investigate the potency of AD-ex to induce neuronal dysfunction, the extract was diluted 1:60 in ACSF, 2-5 µl microliters were filled in a micropipette, and the tip was carefully navigated to neurons with ongoing baseline activity. After recording the baseline, AD-ex was gently pressure-applied to hippocampal neurons. AD-ex strongly induced neuronal hyperactivity (**Fig. 19 B**). The activity increased immediately after application and even escalated during the application in three of the five selected cells of the representative experiment (**Fig. 19 A**). It appears that the activity levels after washout tend to be higher compared to baseline (2,76 vs. 1,81 ≙ mean of all washouts vs. mean of all baselines), even though this is not statistically significant. Nevertheless, this is interesting because it indicates that human-derived Aβ may be more adhesive to the neuronal tissue than synthetic peptides, which could also explain their presumed pronounced toxicity. Persisting hyperactivity further serves as an indirect control experiment, since this phenomenon cannot be explained by acute vehicle application alone.

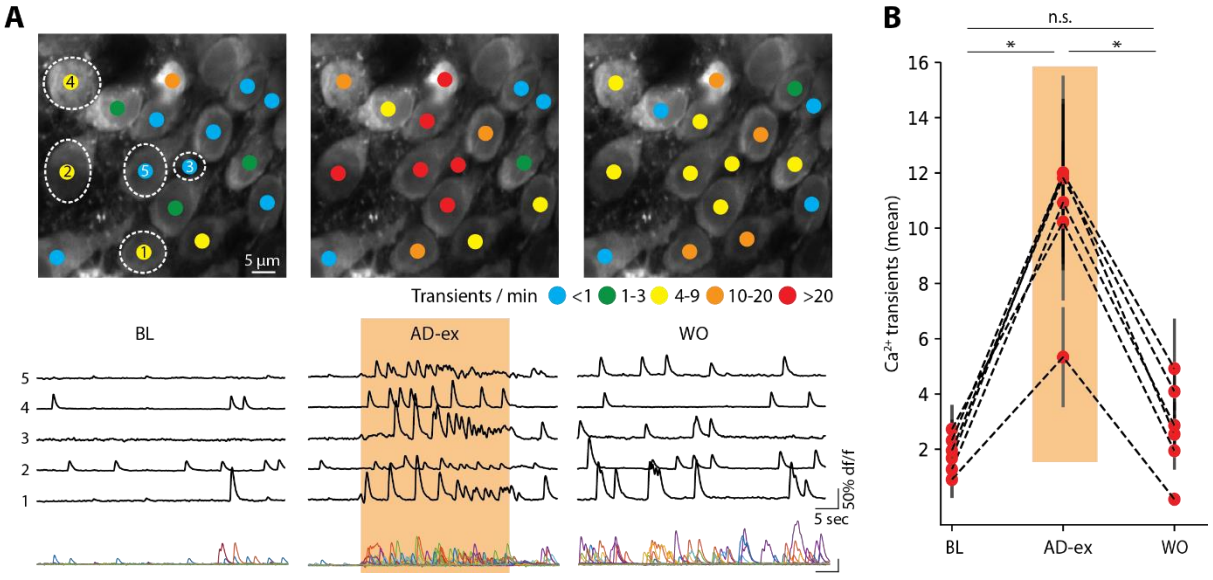

Fig. 19: Aβ-containing brain extract potently triggers neuronal hyperactivity. A) Representative two-photon images with five circled cells (up), corresponding Ca^{2+} traces of selected cells (middle) and overlayed traces of all cells (z = 26) (down) from the respective experiment for baseline, AD-ex application (diluted 1:60, shaded area) and washout. Colored dots represent the number of Ca^{2+} transients per minute during each phase. **B)** Quantification of data shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, AD-ex application and washout. Lines connect dots of one coherent experiment (n = 6). Error bars display SEM. Wilcoxon test (B). * $p < 0.05$, n.s. not significant.

The neuronal dysfunction is activity-dependent

As mentioned earlier in chapter 1.2.3, synthetic Aβ-mediated hyperactivation requires baseline activity (Zott et al 2019). However, it is unclear whether this is a general phenomenon or whether it depends on the neurotoxic potential of a certain Aβ species. In order to answer this question, I repeated the previous experiment on untreated silent slices. I reliably recorded no Ca^{2+} transients during the application of AD-ex (**Fig. 20**), although the same extract potently induced neuronal hyperactivation in treated slices with in vivo-like activity (**Fig. 19**). Therefore, AD-ex-induced neuronal dysfunction also requires a certain level of spontaneous baseline activity similar to that of the synthetic Aβ dimer preparation.

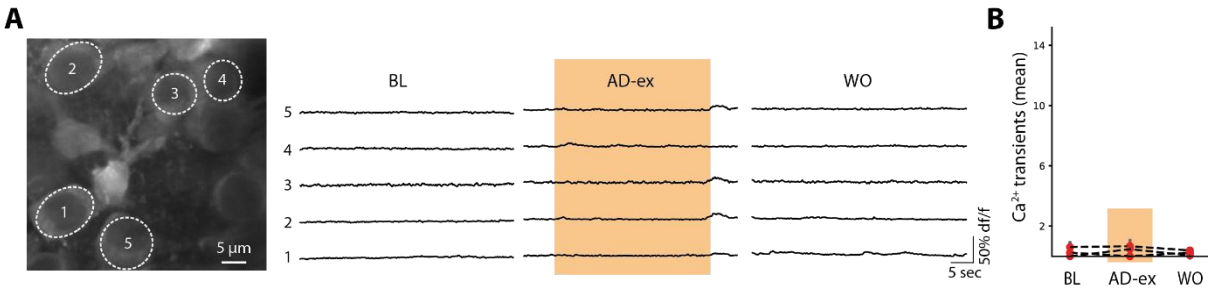

Fig. 20: Neuronal hyperactivation is activity-dependent. A) Two-photon image with five circled cells and corresponding Ca^{2+} traces without transients for baseline, AD-ex application (shaded area) and washout (from left to right). **B)** Summarized data for experiment shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one coherent experiment (n = 3). Reasonably, a statistical test was not performed due to the small sample size. Error bars display SEM.

3.2.2 Immunodepletion of Aβ prevents neuronal dysfunction

So far, I have shown that human-derived AD-ex induces neuronal hyperactivity in hippocampal slices with ongoing activity. However, AD-ex represents homogenized brain tissue from AD patients and therefore contains a myriad of molecules apart from Aβ. Consequently, the hyperactivating effect may not exclusively be attributable to Aβ. For control experiments, Aβ was removed from the solution via immunodepletion (**ID-ex**) with Aβ-binding antibodies. Consequently, ID-ex serves as a vehicle control to test whether AD-ex-induced neuronal dysfunction is caused by Aβ. Notably, AD-ex and ID-ex were obtained from the same patient. ID-ex was equally diluted in ACSF, a few microliters were filled in a pipette, and the vehicle solution was applied to neurons with ongoing baseline activity. ID-ex had almost no effect on

the preceding activity level of the five selected cells of one representative experiment which proved our expectations (**Fig. 21**).

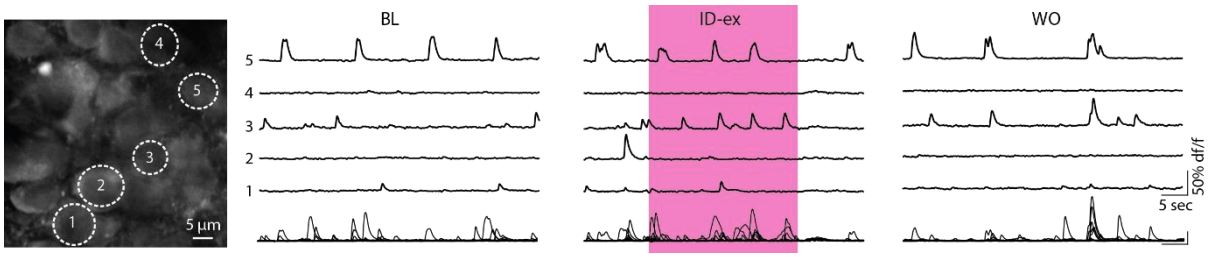

Fig. 21: Immunodepletion of Aβ abolishes the neurotoxic potential of AD-ex. Two-photon image with five circled cells and corresponding Ca^{2+} transients for baseline, ID-ex application (diluted 1:60, shaded area) and washout (from left to right). Below, overlayed traces of all cells from the respective experiment (z = 21).

I plotted the distribution of the activity change from baseline to application (Δ Activation) for all AD-ex and ID-ex experiments on a single cell level. While the majority of neurons revealed either no or only a slight increase of activity during ID-ex application (**Fig. 22 B**), hyperactivation was markedly increased for AD-ex (**Fig. 22 A**). Since the hyperactivating effect was significantly decreased for ID-ex compared to AD-ex (**Fig. 22 C**), I concluded that the neurotoxic potential of AD-ex was clearly attributable to Aβ.

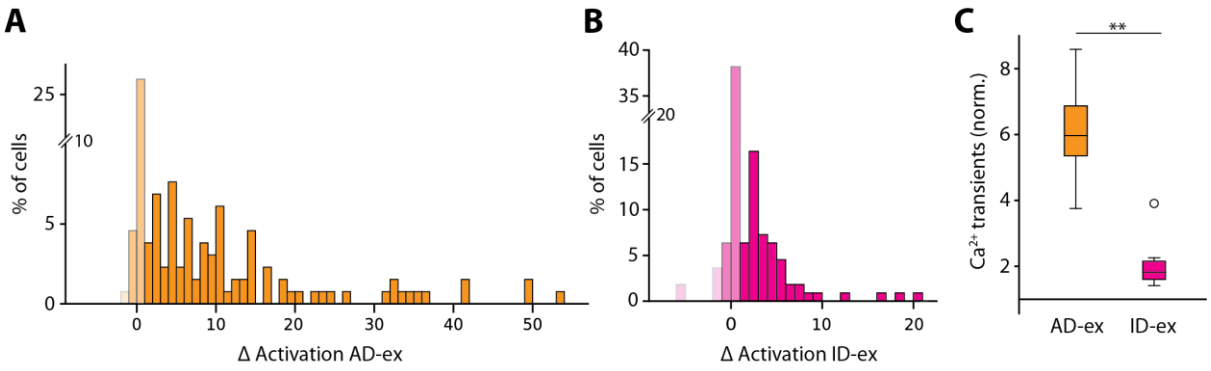

Fig. 22: Human-derived brain extract-mediated neuronal dysfunction is Aβ-dependent. A) Histogram showing the extent of hyperactivation from baseline to AD-ex application (Δ Activation) for data shown in Fig. 19 (z = 131, n = 6). **B)** Same as A) for quantitative ID-ex data (z = 110, n = 6). **C)** Box plots of mean Ca^{2+} transients normalized to baseline for experiments shown in A) and B). MWU test (C). ** $p < 0.01$.

3.2.3 Human Aβ dimers are neurotoxic in non-silent neurons

For synthetic peptides, I showed that the neurotoxic potential depends on the size of the respective Aβ aggregate. But do these results translate to human-derived Aβ? In a further analysis of the AD-ex solution, our partners identified two specific and many larger non-specific Aβ species. Among other things, immunoblotting disclosed sharp bands at 4 and 7 kDa, which are known as the molecular masses of monomers and dimers (Zott et al 2019). The fractions were designated as human-derived Aβ monomers and -dimers, respectively. Both were separated from AD-ex by mass spectrometry and stored at - 80 °C.

Application of highly concentrated human dimers

In a first experiment, I investigated the neurotoxic effect of human-derived Aβ dimers on silent neurons. After staining, human dimers were diluted 1:5 in ACSF and applied to neurons without in vivo-like baseline activity. Similar to synthetic Aβ dimers (**Fig. 3 B**) and AD-ex (**Fig. 20**), human Aβ dimers were equally insufficient to generate activity in silent neurons (**Fig. 23**).

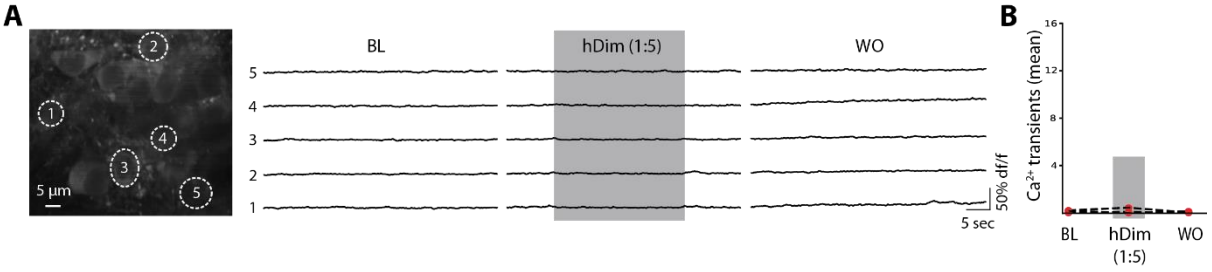

Fig. 23: Human Aβ dimers have no effect on silent neurons. A) Two-photon image with five circled cells and corresponding Ca^{2+} traces without transients for baseline, human Aβ dimer application (shaded area) and washout (from left to right). **B)** Quantitative data for the experiment shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one coherent experiment (n = 3). Reasonably, a statistical test was not performed due to the small sample size. Error bars display SEM.

In order to test the hyperactivating potential of human dimers on spontaneously firing neurons, I added bicuculline and potassium to the ACSF solution again and superfused the slice. For visualization, I plotted the distribution of baseline Ca^{2+} transients for all cells before (**Fig. 24 A**) and after (**Fig. 24 B**) the pharmacological intervention. Remarkably, almost 100 % of the cells were entirely silent under physiological conditions in vitro. The cumulative distribution of the activity levels differed significantly between the two groups (**Fig. 24 C**).

After establishing in vivo-like neuronal activity, I repeated the injection of human Aβ dimers. Noteworthily, this caused a massive gain of activity in all six selected cells with preceding baseline activity (**Fig. 25 A**). The human Aβ dimers application achieved an effect size that outperformed previous observations with other Aβ species (**Fig. 25 B**).

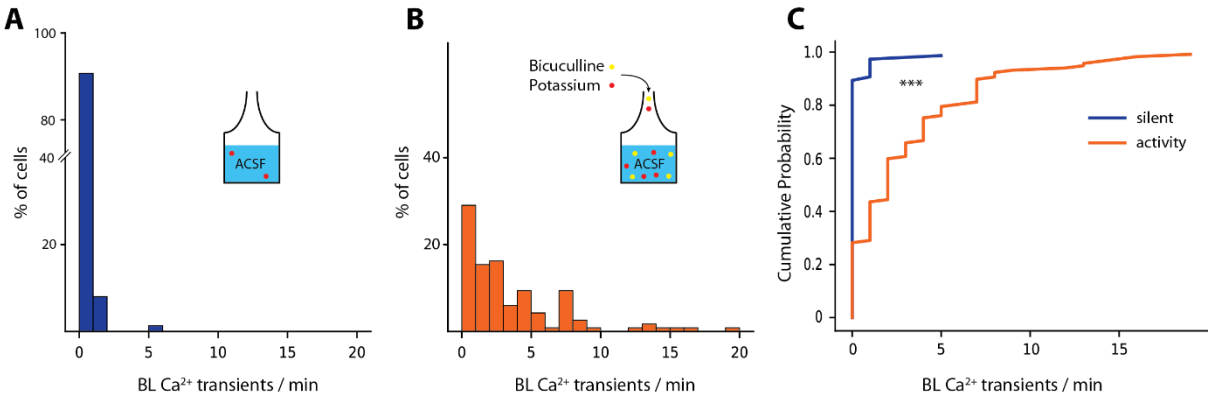

Fig. 24: Pharmacological induction of spontaneous baseline activity. A) Histogram of Ca^{2+} transients during baseline for 75 cells (n = 3) superfused with physiological ACSF. **B)** Same as A) for 117 cells (n = 5) superfused with ACSF with increased potassium and bicuculline concentrations. **C)** Cumulative distribution of the data shown in A) and B). Kolmogorov-Smirnov test (C). *** $p < 0.001$.

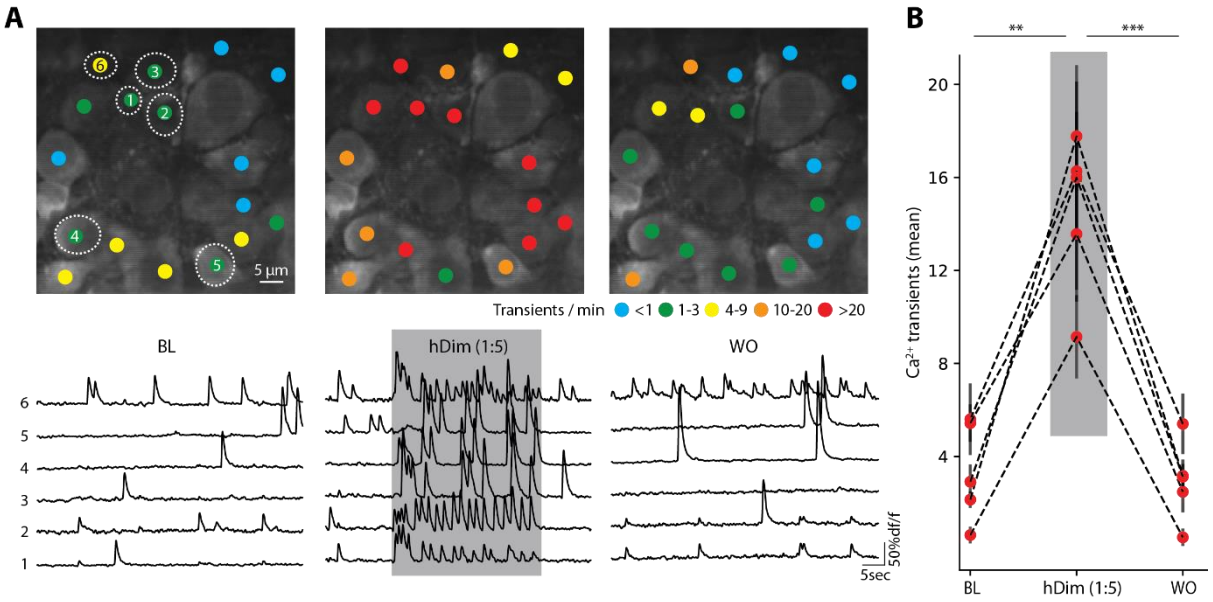

Fig. 25: Concentrated human Aβ dimers extensively induce hyperactivity in neurons with preceding baseline activity A) Representative two-photon images with six labelled cells (up) and corresponding Ca^{2+} traces for baseline, human Aβ dimer application (diluted 1:5, shaded area) and washout (down). Colored dots represent the number of Ca^{2+} transients per minute during each phase. **B)** Quantification of data shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one coherent experiment (n = 5). Error bars display SEM. Paired sample t-test (B). ** $p < 0.01$, *** $p < 0.001$.

Application of highly diluted human dimers

The previous experiment revealed an enormous neurotoxicity of human Aβ dimers. However, occasionally, the application caused giant Ca^{2+} transients leading to an irreversible plateau state of the respective cells that persisted over time. Since overshooting Ca^{2+} influx can cause cell damage, I interpreted this observation as Aβ-induced excitotoxicity (Arundine & Tymianski 2003). Thus, I increased the dilution factor and repeated the experiment with a 1:20 diluted preparation. The application of more diluted dimers reliably triggered neuronal hyperactivity (**Fig. 26 A, B**), indicating that human Aβ dimers remains neurotoxic even at lower concentrations. As expected, the effect decreased compared to the 1:5 dilution (**Fig. 26 C**), however, the effects do not differ significantly most probably due to the disparate distribution of the data. In particular, this semiquantitative dose-effect relationship implies that the extent of hyperactivation is dependent on the concentration of Aβ. This is consistent with the observation that neuronal hyperactivity decreases with increasing distance from the application site (**Fig. 18**).

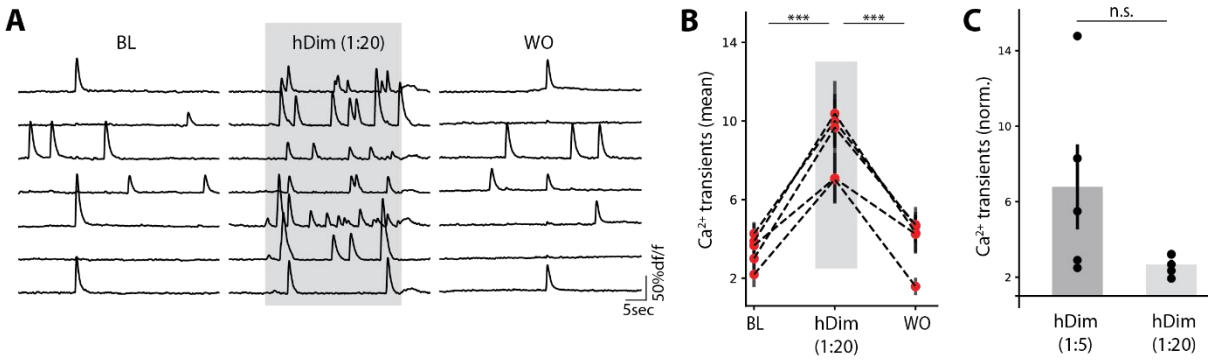

Fig. 26: Human Aβ dimers remain neurotoxic even at lower concentrations. A) Ca^{2+} traces of seven elected cells for baseline, human Aβ dimer application (diluted 1:20, shaded area) and washout of one representative experiment (from left to right). **B)** Quantitative data for the experiment shown in A). Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one single experiment (n = 5). **C)** Bar graph of mean Ca^{2+} transients normalized to baseline for the application of the 1:5 diluted (left) and 1:20 diluted (right) preparation. Error bars display SEM. Paired sample t-test (B). MWU test (C). *** $p < 0.001$, n.s. not significant.

3.2.4 Human-derived- and synthetic Aβ dimers differ in neurotoxicity

Next, I compared the effects of the human Aβ 1:5 preparation and synthetic Aβ dimers. Although it is impossible to calculate the exact concentration of Aβ in the human preparation,

an approximation considering the concentration of the sample and the molecular mass indicated that the solution contains dimers in the lower nanomolar range (Zott 2019). By contrast, synthetic Aβ dimers were applied in a concentration of 0.5 – 1-0 µM, consequently, the human preparation was far less concentrated than the synthetic Aβ dimers solution. Notably, this approach is not appropriate to properly compare the hyperactivity-inducing potential of both agents, nevertheless, a rough, semiquantitative approximation of the different neurotoxic potentials can be made.

I plotted the distribution of the change in activity from baseline to application (Δ Activation) for all cells from both groups (**Fig. 27 A, B**). The magnitude of hyperactivation reached values up to 20 in the synthetic dimer cohort, whereas larger effects up to 40 were achieved with human Aβ dimers. The cumulative distribution of the hyperactivating effects differed significantly between the two preparations (**Fig. 27 C**). Furthermore, I categorized the fraction of cells with (i) hyperactive, (ii) hypoactive, or (iii) unchanged activity during the application for both cohorts as following:

$$\underline{Hyperactivation}:$$

$$(Application - Baseline)_{\,Ca^{2+}transient\,(min)} > 2 \times (Baseline)_{\,Ca^{2+}transient\,(min)}$$

$$\underline{Hypoactivation}:$$

$$(Application)_{\,Ca^{2+}transient\,(min)} < (Baseline)_{\,Ca^{2+}transient\,(min)}$$

For human dimers, 64 % of all 117 cells became hyperactivated during the application (**Fig. 27 E**), compared to only 36 % of the 403 cells in the synthetic cohort (**Fig. 27 D**). Furthermore, only 8 % — in contrast to 16 % in the synthetic dimer cohort — were hypoactivated during human dimer application. This analysis permits one to assume that the latter exhibit a larger potency at even lower concentrations than synthetic Aβ dimers, which is in line with previous evidence (Li & Selkoe 2020). Taken together with my previous results, it seems that the extent of neurotoxicity depends not only on the Aβ size (**Fig. 16 C**), but also on the source of the Aβ peptides (**Fig. 27 C**).

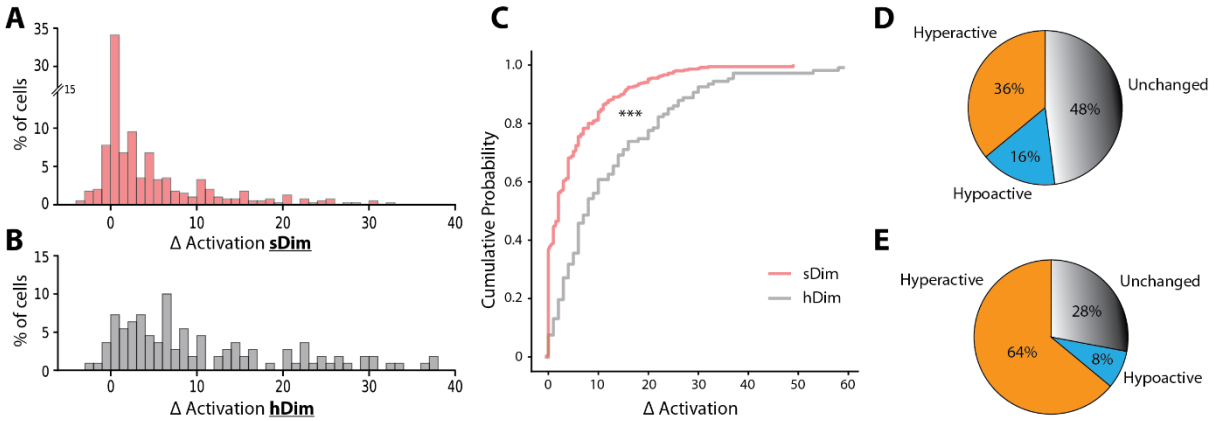

Fig. 27: The extent of neurotoxicity varies between Aβ dimers from different sources. A) Histogram of the neuronal activity increase (Δ Activation) induced by synthetic Aβ dimers for 404 cells. **B)** Same as A) for 1:5 diluted human-derived Aβ dimers for 117 cells. **C)** Cumulative distribution of the data shown in A) and B). **D)**, **E)** Pie charts for categorized data (as defined above) presented in A) and B). Kolmogorov-Smirnov test (C). *** $p < 0.001$.

3.2.5 Human Aβ monomers do not induce neuronal dysfunction

I raised the question whether the size-dependence of neuronal hyperactivation can be transferred to human preparations. It has already been shown that human-derived monomers do not interrupt the induction of synaptic plasticity (Shankar et al 2008), however, it is still unknown whether they also fail to trigger neuronal hyperactivity. In order to screen them for their potential to induce hyperactivity, I adopted the protocol from the previous experiment. Notably, 1:5 diluted human Aβ monomers failed to induce neuronal hyperactivity (**Fig. 28 A**). Pooled quantitative data of monomer experiments (1:5 and 1:20 preparation) revealed a marginally significant effect (**Fig. 28 B**), but this effect was similar to that observed in control experiments, and human Aβ dimer-induced neuronal dysfunction undoubtedly differed from the human Aβ monomer cohort (**Fig. 28 C**). Additionally, the cumulative distribution of the number of Ca^{2+} transients during the application of ACSF, human-derived monomers and dimers, and AD-ex showed that monomers behave similarly to the vehicle, whereas dimers behave similarly to AD-ex (**Fig. 28 D**). The latter is in line with the fact that AD-ex contains much more Aβ dimers than Aβ monomers (Zott et al 2019). Based on this data, I conclude that human Aβ monomers, similarly to synthetic Aβ monomers, are not neurotoxic. Again, this experiment underlines the significance of the Aβ size in determining the corresponding neurotoxic potential.

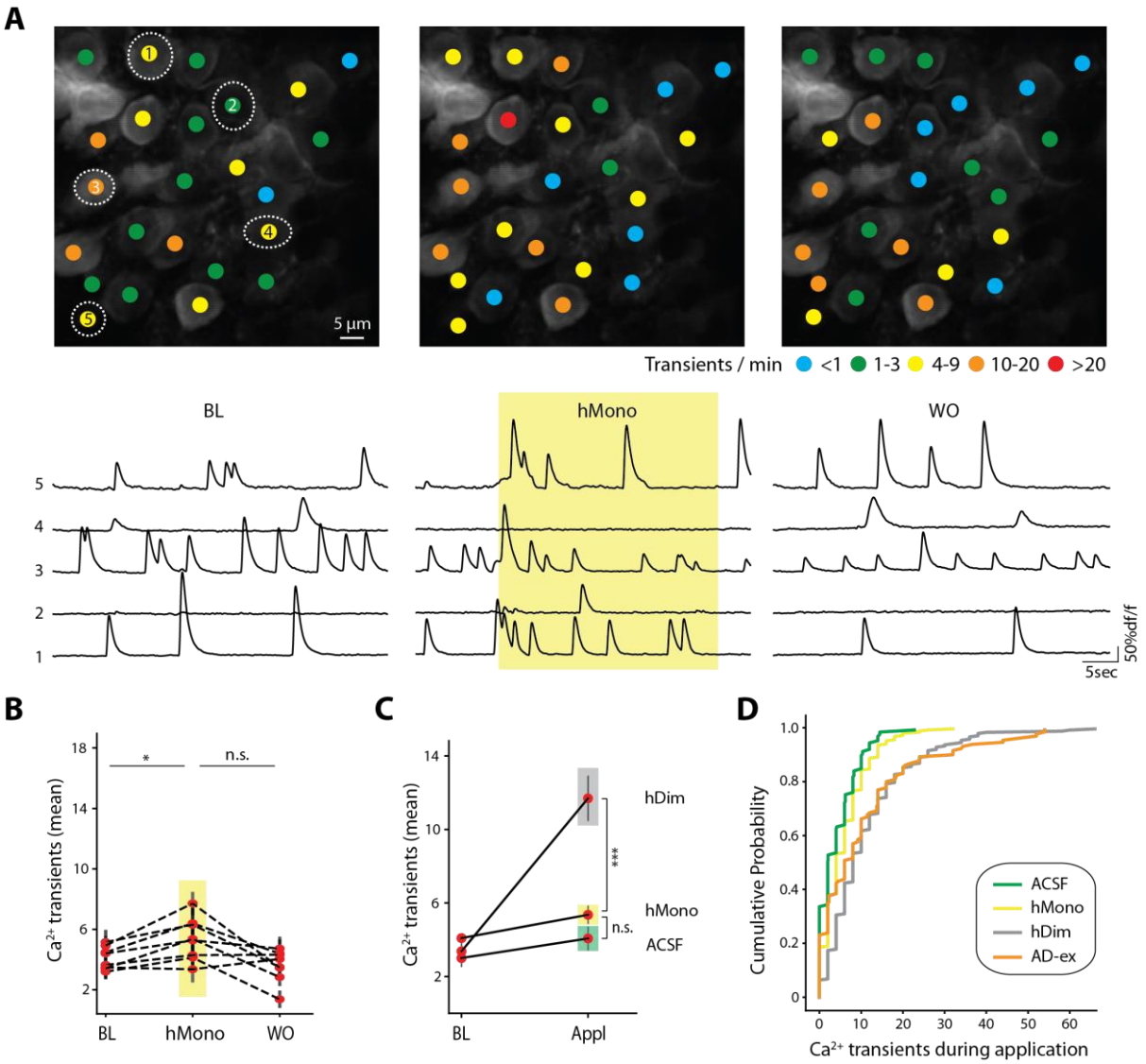

Fig. 28: Human Aβ monomers are not neurotoxic. A) Representative two-photon images with five circled cells and corresponding Ca^{2+} transients for baseline, human Aβ monomer application (diluted 1:5, shaded area) and washout (from left to right). Colored dots represent the number of Ca^{2+} transients per minute during each phase. **B)** Quantification of data shown in A) pooled with experiments with a dilution factor of 1:20. Dots indicate the mean of Ca^{2+} transients per minute for baseline, application and washout. Lines connect dots of one coherent experiment (n = 8). **C)** Summary of data with human Aβ preparations compared to vehicle control. Dots represent means of mean Ca^{2+} transients during baseline and application for experiments with human Aβ dimers (n = 10, grey box), human Aβ monomers (n = 8, yellow bock) and vehicle control (n = 6, green box). Lines connect corresponding dots. Mean Ca^{2+} transients normalized to baseline were statistically tested. **D)** Cumulative distribution of Ca^{2+} transients per minute during the application of ACSF (z = 125), human Aβ monomers (z = 228) and dimers (z = 236) and AD-ex (z = 130). Error bars display SEM. Wilcoxon test (B), MWU test (C). * $p < 0.05$, *** $p < 0.001$, n.s. not significant.

In summary, while some evidence has already indicated that Aβ monomers and dimers may differ in toxicity (Shankar et al 2007), my work provides clear evidence of their ability to cause neuronal dysfunction. My findings are in line with the observation that toxicity increases with Aβ size (Hayden & Teplow 2013) because I found a sharp gain of neurotoxicity from monomers to dimers. Although Aβ monomers were found to be initially nontoxic, they remain nevertheless of major importance because they instantaneously form toxic AβOs (**Fig. 29**).

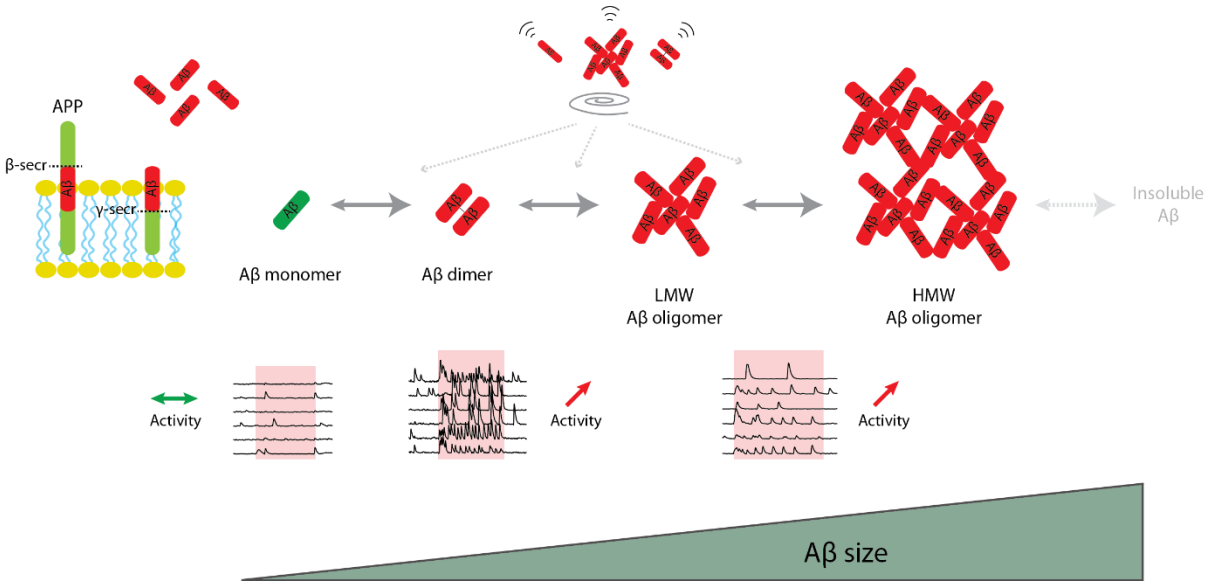

Fig. 29: Abrupt gain in neurotoxicity from Aβ monomers to Aβ dimers and larger Aβ aggregates. Aβ monomers (green) do not induce neuronal hyperactivity in acute hippocampal brain slices. In contrast, Aβ dimers and putative larger oligomers (red) reveal a strong hyperactivating potential. Overall, Aβ toxicity highly depends on the size of the respective Aβ molecule. LMW: Low-molecular-weight, HMW: High-molecular-weight.

4 Discussion

Previous AD research identified AβOs, instead of Aβ plaques, as the main neurotoxic agent and pinpointed neuronal hyperactivity as an early AD-related dysfunction. AβOs were found to directly cause hyperactivity and notably, the reduction of this dysfunction had beneficial effects on cognition. AβOs vary in size, therefore it is necessary to systematically investigate their hyperactivity-inducing potential.

4.1 Aβ size dependence of neuronal toxicity

Aβ monomers

My systematic investigation of different Aβ species disclosed that synthetic and human Aβ monomers do not induce neuronal hyperactivity in acute hippocampal slices (**Fig. 10, 28**). This is in line with previous results (Shankar et al 2007, Walsh et al 2002, Klyubin et al 2005, Hartley et al 1999, Cizas et al 2010) and with in vivo findings from the Konnerth group (Zott et al 2019). In fact, Aβ monomers are present in the brain and CSF of healthy individuals (Shoji 2002) and may even have a protective role, for instance due to an antimicrobial property or the ability to promote recovery from brain injury (Jeong et al 2022). Thus, Aβ 1-42 monomers were found to support the survival of neurons by protecting them from excitotoxicity (Giuffrida et al 2009) and by preventing apoptosis (Guglielmotto et al 2014). Moreover, monomers interact with growth factor receptors to provide energy supply (Giuffrida et al 2015) and to stimulate aerobic glycolysis in response to metabolic stressors (Santangelo et al 2021). Conversely, the inhibition of Aβ release causes neuronal loss that can be prevented by adding Aβ to neuronal cultures (Morley et al 2019).

Aβ dimers

My thesis suggests that Aβ dimers are the lowest-n-molecular synaptotoxic AβOs. Human Aβ dimers caused neuronal dysfunction in vitro. Notably, identical results were previously reported by our group in the intact neuronal network in vivo (Zott 2019, Zott et al 2019, Busche et al 2012). Together with evidence that Aβ toxicity follows rank order from monomers to larger assemblies (Hayden & Teplow 2013), this work also suggests an (early) erratic

increase of Aβ size-related neurotoxicity from monomers to dimers. This is in line with findings that naturally secreted Aβ dimers and trimers, but not monomers, induce hippocampal synaptic spine loss in organotypic brain slices. The effect is reversible and can be blocked by Aβ-specific antibodies and modulators of Aβ aggregation (Shankar et al 2007). Remarkably, the potency of trimers to inhibit LTP is higher than that of Aβ dimers (Ono et al 2009), suggesting that dimers may be the smallest but not the most toxic form of AβO (Townsend et al 2006). Further investigations showed that the LTP disruption caused by human AβOs is specifically attributable to dimeric human Aβ (Shankar et al 2008) and, consistently, Aβ dimer-containing CSF from AD patients was found to impair synaptic plasticity (Klyubin et al 2008). Finally, the Aβ size dependence of neuronal toxicity is supported by intervention studies. It was shown that the binding of Aβ dimers, but not monomers, ameliorates the plasticity-disrupting effects of Aβ (O'Nuallain et al 2011); similar results were achieved in passive immunization studies (Klyubin et al 2008).

In this work, Aβ-induced neuronal dysfunction was studied by acute bolus application of an Aβ-containing solution to CA1 pyramidal neurons. The Aβ effect was limited to the application period and the ability to induce neuronal hyperactivity was assessed as neurotoxic. This approach allows the systematic screening of Aβ molecules in a high-throughput assay, however, it does not represent the natural course of AD disease, in which neurons are continuously exposed to Aβ during the progression of AD. Additionally, Aβ levels in the brain rise relatively slowly over many decades (Burnham et al 2020). Therefore, it can be assumed that the brain develops compensatory mechanisms during disease progression (Bobkova & Vorobyov 2015) that interfere with Aβ-induced neuronal hyperactivation and are not considered in the respective approach.

Aβ oligomers

In my experiments, AD-ex and Aβ aggregates formed by aggregation of synthetic monomers — probably containing multiple different AβOs — exhibit severe neurotoxic potential. While Aβ monomers were not potent in their native form, I identified them as an important substrate for the oligomerization to toxic AβOs. My findings are in line with previous reports showing an increase of toxicity from monomers to dimers and smaller AβOs (Ono et al 2009). Furthermore, AβOs formed by the aggregation of monomers caused impaired recognition

memory and memory consolidation after they were injected into the brain ventricle of mice, while no effect was observed for freshly dissolved monomers (Balducci et al 2010). Incubated Aβ monomers were also found to be toxic in organotypic tissue cultures (Lambert et al 1998, Picone et al 2009). Overall, although Aβ monomers appear to be nontoxic or even protective, they are of major importance because they obtain neurotoxicity through aggregation to AβOs.

It was beyond the scope of this work to determine the exact size of the Aβ aggregates, however, our collaborators showed that solved Aβ 1-40 monomers form thioflavin-positive aggregates within a few hours, with a rapid rise in the first 60 minutes (Rauth et the 2016). The application of the incubated monomer solution through a patch pipette became more difficult or even impossible after about 60 minutes and I observed dense sludge blocking the pipette tip. Therefore, I reasoned that larger, insoluble assemblies had formed. Since the pressure application was again facilitated after centrifugalizing the solution, I further assumed that the toxic supernatant mainly consists of soluble LMW AβOs. However, verifying this is also beyond the scope of this article. As noted above, the term “oligomer” is used to refer to a broad spectrum and the exact identity of toxic AβOs is still unknown (Benilova et al 2012).

The toxicity of larger AβOs is controversially discussed in the field, and importantly, AβOs are not uniformly classified by size. Scientists commonly divide them into lower- and higher-molecular weight AβOs. Cline and colleagues drew the line at 50 kDa and reported that HMW oligomers are more critical for AD-related pathology than LMW oligomers (Cline et al 2018). In line with this observation, HMW AβOs disturb the integrity of the plasma membrane to a higher extent than LMW AβOs (Yasumoto et al 2019). Conversely, evidence suggests that mainly LMW AβOs are promising candidates for mediating neurotoxicity in AD (Sengupta et al 2016), and it is assumed that LMW AβOs are more toxic than larger AβOs (Yang et al 2017). Further studies are faced with the challenge of combining structural analysis of (stabilized) AβOs with toxicity assays, such as neuronal dysfunction, in the same experiment in order to discover further toxic AβOs besides Aβ dimers.

Fibrils and Plaques

Although their size prohibits direct application via a glass pipette and I could not investigate the toxic effects of direct fibril application, multiple lines of evidence indicate that Aβ fibrils are less toxic than oligomers (Verma et al 2015, Shankar et al 2008). As previously mentioned, normal cognition can be observed in elderly with severe AD pathology such as plaques (Sengupta et al 2016), consequently, plaque burden has been found to be a poor predictor of cognitive decline (Hardy & Selkoe 2002). In addition, a mutation implicated in the pathogenesis of FAD has been discovered that is independent of insoluble forms of Aβ (Inayathullah & Teplow 2011). Finally, facilitating fibrillogenesis attenuates the suppression of LTP caused by AβOs in brain slices, also pointing to a more innocent role of Aβ deposits (Bieschke et al 2012). Together, these findings triggered the shift of attention that is described in detail in chapter 1.2.

4.2 Human-derived vs. synthetic Aβ

In this work, commercially available purified synthetic Aβ and human-derived Aβ from AD patients were used. Dimers of both preparations caused neuronal dysfunction, while monomers had no effect (**Fig. 16, 28**). Further, AD-ex (**Fig. 19**) and synthetic Aβ aggregates (**Fig. 13, 15**) — both putatively containing a mixture of different Aβ molecules — induced neuronal hyperactivity. It is important to mention that AD-ex is a heterogeneous solution containing numerous grey and white matter components in addition to Aβ. However, ID-ex, which was not bioactive in my assay (**Fig. 22**), differed from the hyperactivity-inducing whole brain extract only by the immunodepletion of Aβ. This demonstrates that the effect of the brain extract can only be mediated by Aβ. One conclusion of this work is that the effect of Aβ depends more on the Aβ size and less on the source of the respective preparation (Li et al 2011). Though, human Aβ dimers appear to be more potent than synthetic dimers (**Fig. 27**), which is in line with recent evidence (Li & Selkoe 2020). One obvious explanation might be an aberrant peptide concentration but I applied synthetic dimers from 0.5 – 1-0 µM, whereas human dimers were diluted 1:5 to a low nanomolar concentration. Since a lower concentration of the human preparation had a stronger effect than the synthetic dimer, I argue that human-derived Aβ is more toxic. Consistently, subnanomolarly-concentrated Aβ di-

mers from AD patients caused neuritic degeneration in cultured neurons, while synthetic dimers were far less effective (Jin et al 2011). AD-related changes were caused by 0.1 µM of synthetic AβOs in a 3D in vitro cell culture model (Zhang et al 2014) and 20 µM of a synthetic Aβ peptide fragment (Takashima et al 1998). Similar results were reported from experiments comparing the potency of synthetically- and naturally-secreted Aβ. The latter is produced by transfected culture cells (Reed et al 2011). In vivo, an nM-concentrated extract (Varshavskaya et al 2022) from the brains of aged APP transgenic mice was injected in young transgenic AD mice and induced AD pathology (Bolmont et al 2007); similar results were achieved with a 100 µM solution of synthetic AβOs (Selenica et al 2013). Although these studies are heterogenous and use different models to measure different toxicity-related endpoints, the observation that synthetic preparations must be more concentrated to obtain significant effects is entirely consistent with my experiments.

It seems that Aβ molecules from different sources have different properties (Varshavskaya et al 2022), but the reasons may be manifold. For instance, sophisticated solid-state nuclear magnetic resonance spectroscopy with enhanced sensitivity found obvious structural differences between synthetic and human-derived Aβ fibrils indicating that, at first glance, identical and comparable Aβ molecules may be different on a molecular level (Wickramasinghe et al 2021). Furthermore, synthetic- and human-derived Aβ exhibit a different peptide stability, which may be of major importance, depending on the experimental protocol (Moore et al 2009). This can be explained, at least in part, by differences in the production of synthetic Aβ, it has even been shown that Aβ from different batches are not identical (Varshavskaya et al 2022). Aβ deposited in the human brain physiologically undergoes several posttranslational modification steps such as phosphorylation, which might alter its toxicity (Barykin et al 2017). Remarkably, Aβ with certain posttranslational modifications was shown to impair neuronal viability via completely different mechanisms indicating that human-derived Aβ may mediate toxicity through multiple pathways (Grochowska et al 2017). Human-derived Aβ is also naturally imprinted and influenced by components from the extracellular compartment, such as ions. Copper bound to Aβ has been shown to boost the neurotoxicity and facilitates the aggregation of Aβ (Ayton et al 2013), moreover, also zinc ions tightly interact with Aβ influencing its assembly and degradation (Stelmashook et al 2014). Overall, human-derived Aβ aggregates are mainly affected by the cellular microenvironment, while respective modifications

are not considered in synthetic preparations, which can at least partially explain the different neurotoxic potentials.

Another explanation for the differences in toxicity of the synthetic and human-derived dimers is the cross-linking at different sites. Mass spectrometry showed that the human dimer preparation used in this work mostly contains cross-linked dimers, with the majority forming a bond between an aspartic acid in position 1 and a glutamic acid in position 22 (Brinkmalm et al 2019). By contrast, the synthetic Aβ dimers consist of two Aβ peptides that form a disulfide bond after the amino acids in position 26 are replaced by a cysteine. Finally, the cross-linking of Aβ is strengthened under in vivo conditions and stabilizes Aβ aggregates (Al-Hilaly et al 2013).

4.3 Mechanism of AβO-mediated hyperactivation

In order to affect cellular processes, Aβ must interact with neuronal tissue. Since monomers and dimers differ in their shape (O'Malley et al 2014), this may cause a different neurotoxic potential. Indeed, AβOs were found to strongly bind to cell membranes in contrast to monomers (Narayan et al 2013, Sarkar et al 2013). Aβ interacts with the lipid bilayer in various ways: by electrostatic interaction with membrane lipids and/or via membrane receptors like the low-density lipoprotein receptor, the low-density lipoprotein receptor-related protein 1, acetylcholine receptors, and metabotropic glutamate receptor 5 (Wiatrak et al 2021). The plasma membrane consists of sphingolipids and it has been found that sphingomyelin- and cholesterol-enriched areas are prone to interact with Aβ (Devanathan et al 2006). Larger Aβ sizes are thought to carpet the membrane, and organized forms may even be integrated in the membrane forming pores (Bharadwaj et al 2018). The binding of Aβ triggers signal transduction cascades (Wiatrak et al 2021), and even the incorporation of Aβ into the intracellular space (Kanekiyo et al 2014). Accordingly, the inactivity of Aβ monomers may be due to their lack of binding to the plasma membrane and/or their preserved mobility without significantly interfering with membrane proteins (Chang et al 2018).

How do AβOs cause neuronal hyperactivity? AβOs alter the E/I balance (Vico Varela et al 2019, Busche & Konnerth 2015, 2016), but whether an increased excitation, or a disinhibition, or a combination of them are relevant for the observed neuronal hyperactivity remain enigmatic.

We demonstrated that the Aβ effect is dependent on the glutamate uptake (Zott et al 2019), which may also explain why the neuronal hyperactivity observed in my experiments is activity-dependent. Glutamate homeostasis is tightly regulated and depends on the dynamic equilibrium between presynaptic release and postsynaptic transmitter clearance. During an action potential, glutamate in the synaptic cleft rises from nano- to millimolar values in the subsecond range and is immediately cleared to limit the activation to only a subunit of synapses (Murphy-Royal et al 2017). The reuptake of glutamate from the synaptic cleft is mainly mediated by astrocytes via glutamate transporters. Among them, the astroglial excitatory amino acid transporter 2 (EAAT2) — the rodent counterpart is called glutamate transporter 1 (GLT-1) — is the major glutamate transporter in the brain (Andersen et al 2021). Interestingly, EAAT2 was shown to be reduced in quantity (Zumkehr et al 2015) and activity (Masliah et al 1996) in AD, and dysfunctional transporters were previously detected in the vicinity of Aβ plaques (Hefendehl et al 2016). Although there was already evidence that AβOs impair the reuptake of glutamate (Li et al 2009), until recently, it was unclear how Aβ affects glutamate reuptake. However, our group has collected data that provide a possible mechanistic explanation. In our experiments, we showed that (i) local application of Aβ dimers evoked an increase of fluorescent glutamatergic signals (Unger et al 2021), (ii) the glutamate reuptake inhibitor threo-β-benzyloxyaspartic acid (TBOA) induced neuronal hyperactivity equivalent to Aβ, and (iii) a GLT-1 antagonist and a GLT-1 cross-linking antibody had hyperactivity-inducing potential (Zott et al 2019). In this context, it is important to mention that EAAT2 laterally diffuses within the plasma membrane of astrocytes and that prohibiting that mobility via a cross-linking antibody directly affects the postsynaptic signal, suggesting that the motion of glutamate transporters is essential for a proper function (Murphy-Royal et al 2015). Based on this remarkable finding and our own results, we argue that small AβOs bind to the neuronal membrane and perturbs the lateral diffusion of glutamate transporters. Consequently, the clearance of glutamate from the synaptic cleft is impaired resulting in a prolonged excitation and ultimately neuronal hyperactivation (Zott et al 2019, Zott 2019). Furthermore, this model provides a possible explanation for the diverging neurotoxic potential of monomers that do not bind to the cellular membrane and dimers. Since Aβ-mediated synaptic loss requires activity of glutamatergic excitatory receptors (Shankar et al 2007), which suggests that Aβ-induced reuptake inhibition necessitates a certain baseline synaptic transmission, this work directly

supports the glutamate reuptake inhibition hypothesis because Aβ did not affect silent neurons with no ongoing activity (**Fig. 3, 20, 23**). In consequence, it seems likely that only after the induction of (glutamatergic) baseline transmission (**Fig. 8, 24**), Aβ becomes able to inhibit the reuptake of released glutamate leading to neuronal hyperactivation.

Disinhibition by a degradation of inhibitory inputs might further aggravate neuronal hyperactivity and indeed, interneuronal dysfunction was identified as a contributor to cognitive dysfunction in AD (Palop & Mucke 2016). APP transgenic mice have altered subunit compositions of ion channels in inhibitory interneurons (Verret et al 2012). Furthermore, alterations in the sensitivity of $GABA_A$ receptors were identified in AD brains (Limon et al 2012) and the concentration of GABA was decreased (Gueli & Taibi 2013). Though, the precise mechanism of how different Aβ molecules affect interneurons remains to be elucidated.

4.4 Activity dependence of Aβ-induced dysfunction

Aβ dimers were initially ineffective in inducing neuronal hyperactivity in untreated silent hippocampal slices, in contrast to in vivo. One obvious difference between the two assays is the ongoing spontaneous baseline activity in vivo, which is almost absent in hippocampal slices (**Fig. 3**). This drop in activity is probably due to the cutting of neurites during slicing (Okamoto et al 2014), which abolishes excitatory afferent inputs to CA1 pyramidal neurons. I pharmacologically induced stable in vivo-like activity levels in vitro, in order to establish a high-throughput in vivo-like slice assay to study the hyperactivating effects of different Aβ species under controlled conditions.

Many studies of AD-related alterations are performed in organotypic brain slices. Cultured neurons reveal a significant spontaneous firing rate, which is similar to an in vivo network. This is because neurites regrow during cultivation and the network undergoes a self-restoring rewiring (Okamoto et al 2014). Activity patterns were found to vary between cultured hippocampal and cortical neurons (Charlesworth et al 2015) and cultures from different origins. While rodent cultures develop robust activity patterns after just ten days, human cultures require four times longer to form considerably weaker networks (Napoli & Obeid 2016). In cultured mouse and rat cells, AβOs trigger synapse loss (Shankar et al 2007) and cell death

(Lambert et al 1998); synaptophysin expression, whose level correlates with cognitive function in AD (Sze et al 1997), is reduced by AβOs even in human cell cultures (Sebollela et al 2012). Moreover, increased postsynaptic calcium concentrations were found in rat cerebral cortical cell cultures that are induced through the activation of NMDA receptors by AβOs (Ferreira et al 2012), and incubating cultured cells with AβOs was found to trigger neuronal hyperactivity (Ciccone et al 2019). Remarkably, Aβ 1-42 reduced the neuronal survival in cultures only after several days of incubation in a dose-dependent manner (Pike et al 1991); within an equivalent assay, Aβ monomers did not induce synapse loss, in contrast to small AβOs (Shankar et al 2007). Both of these cell culture findings are in line with my in vitro findings that initially harmless Aβ monomers can obtain neurotoxicity. Overall, tissue cultures are appropriate to study Aβ toxicity and represent an attractive alternative to inert acute slices, for example due to their spontaneous neuronal activity under physiological conditions. However, in contrast to cell cultures, freshly prepared slices exhibit an intact microenvironment with microglia and astrocytes, which has been suggested to play an important role in AD (Fuso 2018). Together, methodological finesses of each assay must be considered by interpreting and especially by comparing respective results.

It seems that the most valuable approach for studying the effect of Aβ peptides on spontaneously firing neurons is the intact in vivo organism. Though, under isoflurane anaesthesia used for in vivo experiments, the spontaneous baseline activity is reduced (Greenberg et al 2008) and varies between different brain areas, e.g. cortical layer 2/3 neurons exhibit low spontaneous activity in contrast to layer 5 neurons. Remarkably, Aβ dimers induce neuronal hyperactivity in layer 5 neurons but not in layer 2/3 neurons in vivo (Zott 2019), further strengthening the notion that a certain level of ongoing activity is necessary for Aβ-caused neuronal dysfunction. Furthermore, the firing rate was found to vary during the development of the organism indicating that the neuronal activity level changes with age (Che & De Marco García 2021, Radulescu et al 2021), but it is still unclear how this might affect the susceptibility to Aβ-induced dysfunction. Therefore, my in vitro assay is a valuable alternative or complement to investigate Aβ-induced neuronal dysfunctions under more controlled conditions.

Clinical implications

AD pathology affects certain brain areas in a specific order. The medial temporal lobe with the hippocampus and brain regions such as posteromedial cortical areas, encompassing the precuneus and posterior cingulate cortex, are affected by initial AD-related changes (Sperling et al 2010). Scientists also found that early Aβ accumulation occurs within these areas (Palmqvist et al 2017). Since the first cognitive AD-related deficit is impaired memory, it appears logical that the hippocampus as the main area for memory formation is affected by the earliest neuropathological changes. However, it is unclear why certain areas of the brain are more prone to AD-related brain damage than others. My observation that ongoing neuronal activity makes neurons susceptible to Aβ-induced dysfunction may provide an explanation why different brain areas are unequally affected by AD pathology: these areas are strongly interconnected in the neuronal network indicating pronounced levels of neuronal activity (Utevsky et al 2014, Myers et al 2014, Sperling et al 2010, Huijbers et al 2015).

An increase of the firing rate was also observed in rats (Wilson et al 2005) and mice (Lerdkrai et al 2018) during aging; the aging brain leans toward an excitatory imbalance (Mattson & Arumugam 2018). Hyperactivity was even reported to be accompanied by brain hypermetabolism and an increase of the Aβ burden in cognitively normal elderly subjects (Oh et al 2014). However, it remains unknown whether the rising brain activity in elderly is a general phenomenon that causes a common, age-related cognitive decline, or whether it represents a compensatory mechanism to maintain cognition (Sperling et al 2009).

A positive correlation between neuronal activity and Aβ secretion/deposition, suggesting that hyperactivation may directly drive AD pathology, leads to a vicious cycle of neuronal hyperactivity and Aβ pathology (Buckner et al 2005, Korzhova et al 2021). This further explains why the susceptible brain areas mentioned above develop severe AD pathology. Synaptic activity was shown to regulate the level of Aβ in the interstitial fluid in vivo (Cirrito et al 2005) with more Aβ with increasing activity levels (Kamenetz et al 2003). Consistently, neuronal hyperactivity is linked to more amyloid in transgenic AD mice (Palop et al 2007) and determines the vulnerability to Aβ deposition (Bero et al 2011). These results highlight that the activity-dependent self-potentiating mechanism is aggravated by rising Aβ concentration, which in turn illustrates that an early diagnosis is critical for a successful therapeutical intervention (Rasmussen & Langerman 2019). The activity-dependence of Aβ-induced dysfunction may

also explain the beneficial effects of inhibitory drugs like levetiracetam (Bakker et al 2012, Bakker et al 2015), benzodiazepines (Busche et al 2015), and memantine (Robinson & Keating 2006). However, recently, levetiracetam unexpectedly had no effect on cognitive function in AD patients, (Vossel et al 2021), a further similar study is still on the way (Sen et al 2021). While the first two drugs are still being used in animal experiments and clinical trials, the glutamate receptor antagonist memantine is one of the few permitted drugs for the symptomatic treatment of AD patients and further supports the glutamate reuptake inhibition hypothesis.

4.5 Open questions

Glutamate toxicity

AβOs inhibit the reuptake of glutamate and we assume that this causes glutamate toxicity. In line, the knockout of GLT-1 in mice implicates excessive neuronal activity and reduced animal survival (Tanaka et al 1997) and in addition, prolonged infusion of TBOA causes cell death in hippocampal slices (Bonde et al 2003). Both interventions interfere with the reuptake of glutamate and result in cellular toxicity. Mechanistically, due to the resulting glutamate spillover, the transmitter diffuses out of the synaptic cleft and activates extrasynaptic NMDA receptors (Wang & Reddy 2017). Extrasynaptic NMDA receptors are enriched in the GluN2B subunit (Papouin & Oliet 2014), which is thought to mediate synaptotoxicity (Rammes et al 2017). The toxicity is further aggravated as Aβ induces the internalization of synaptic NMDA receptors (Snyder et al 2005) indicating that more glutamate binds to the extrasynaptic counterpart. However, extrasynaptic NMDA receptors do not only mediate damaging signals, but they are also recruited during bursts of activity under physiological conditions (Harris & Pettit 2008). Excessive glutamate signalling has been shown to affect neuronal viability and cause cell death (Lewerenz & Maher 2015); further, it has been linked to an altered neuronal Ca^{2+} homeostasis (Mattson et al 1992). One possible mechanism is that the excessively activated Ca^{2+} -permeable NMDA receptors cause an overshoot of Ca^{2+} and ultimately lethal influx into the cells (Choi 1992). Finally, the concept of (chronic) glutamate toxicity in AD pathology rationalizes the use of the NMDA receptor antagonist memantine. Although NMDA receptor activity is essential for neuronal survival and blocking it produced severe clinical side effects

(Lipton 2004), memantine proved to be beneficial to AD patients, most likely because of its tightly tuned receptor affinity (Wenk 2006). Overall, chronic glutamate toxicity triggered by Aβ-mediated reuptake inhibition probably contributes to the neuronal excitotoxicity that leads to neurodegeneration in AD. Therefore, NMDA receptor-mediated glutamatergic transmission is an auspicious therapeutic target (Lipton 2004).

Inhibition of Aβ monomer aggregation

I have demonstrated that initially nontoxic Aβ monomers acquire hyperactivity-inducing potential through incubation. My assumption is that the monomers oligomerize to neurotoxic AβOs, but this must be confirmed in further experiments. Though, a similar aggregation assay showed the formation of thioflavin-positive, β-sheet-containing aggregates (Rauth et al 2016), i.e., fibrils. Due to the nature of the Aβ aggregation process, it must be assumed that oligomers arise as an intermediate step (Finder & Glockshuber 2007). Remarkably, this finding has therapeutical implications. The inhibition of monomer aggregation could prevent the formation of toxic Aβ species in the brain. Indeed, major efforts have already been undertaken to develop inhibitors of Aβ monomer aggregation. This turned out to be challenging because protein-protein interactions are difficult to target, and the shape of proteins is inherently dynamic and plastic (Nie et al 2011). It was shown in vitro that carbenoxolone hinders the Aβ 1-42 monomer aggregation (Sharma et al 2017). Furthermore, cyclohexanehexol stereoisomers inhibit the aggregation of Aβ peptides in transgenic AD mice in vivo with positive effects on cognition (McLaurin et al 2006). The same molecule was tested in a phase 2 clinical trial but the primary efficacy outcomes were not met, most likely due to a small sample size (Salloway et al 2011). However, more clinical trials are already underway (Chowdhury et al 2019). Our collaborating partners recently developed a high-affinity Aβ-binding anticalin that can block the aggregation of Aβ 1-40 monomers (Rauth et al 2016). Prospectively, I plan to investigate whether blocking the aggregation of Aβ monomers by the anticalin protein prevents the acquisition of neurotoxic potential.

4.6 Conclusion

In my thesis, I systematically investigated the hyperactivating effect of various sizes of Aβ aggregates from different sources in an in vitro mouse model. I studied synthetic and human-derived Aβ monomers and dimers, Aβ-containing brain extract from AD patients, and aggregated synthetic Aβ. First, I found that Aβ-mediated hyperactivation requires a certain level of spontaneous baseline activity. This compelling evidence supports the mechanism of an Aβ-mediated glutamate reuptake inhibition, leading to a vicious cycle of Aβ-dependent neuronal hyperactivation (Zott et al 2019, Zott 2019). Second, neuronal toxicity is a function of the Aβ size. Dimers proved to be neurotoxic, whereas monomers did not induce neuronal hyperactivity. This was observed equally for synthetic and human preparations. Third, synthetic Aβ 1-40 monomers gain neurotoxic potential through oligomerization to larger species. Within the context of the Aβ aggregation cascade, this work helps to identify the main toxic forms of Aβ and even provides targets for immunotherapy and other therapeutic approaches.

5 Publications

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