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BACE inhibition-dependent repair of Alzheimer's disease pathophysiology

Aylin Derya Keskin-Dargin

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Vorsitzende/r:	Prof. Dr. Thomas Korn
Betreuer:	Prof. Dr. Arthur Konnerth

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1.	Prof. Dr. Helmuth Adelsberger
2.	Prof. Dr. Johann J. Förstl
3.	Prof. Dr. Martin Kerschensteiner

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and presents a major challenge for aging societies. The widely adopted amyloid cascade hypothesis places amyloid-beta (Aβ) at the center of a pathology initiating cascade. Therefore, Aβ presents a promising therapeutic target. The β-secretase 1 (β-site of APP cleaving enzyme - BACE1) is involved in the generation of Aβ, and by pharmacologically blocking its activity, excessive cerebral Aβ accumulation can be hindered. Powerful BACE1 inhibitors have been tested preclinically and shown to clear cerebral Aβ burden in transgenic animals effectively. A limitation of these antecedent studies was that although BACE1 inhibitors have been shown to reduce cerebral Aβ levels, it was unclear whether they would improve brain circuit function. Moreover, preclinical and clinical data raised major concerns about BACE1 mechanism-based side effects on cognition. In order to reveal how a BACE1 inhibitor treatment affects neuronal circuit activity in AD, two-photon and widefield calcium imaging techniques were combined in an APP23xPS45 mouse model. For this purpose, neuronal activity on the cellular level and slow-oscillations on the long-range circuit level were assessed. In addition, a behavioral essay to evaluate the mice's cognitive status was performed. This work offers experimental *in vivo* evidence that BACE1 inhibition is beneficial for brain circuit activity in an AD mouse model mimicking the early amyloidogenic phase of AD. The experiments revealed that chronic BACE1 inhibition recovered neuronal hyperactivity, repaired long-range coherence and memory defects. Most strikingly, brain activity was rescued in aged animals despite their extensive Aβ pathology, suggesting that neurons were still viable and no widespread neurodegeneration occurred. Unraveling the mechanism of BACE1 inhibition-dependent functional recovery, this effect was attributed to the reduction of toxic prefibrillar Aβ species surrounding

neuritic Aβ plaques. By reintroducing those Aβ species in treated animals, the reemergence of dysfunctions on the cellular and long-range level were observed. Addressing concerns about BACE1 inhibitor-related side effects, no abnormal activity patterns in treated transgenic and wild-type animals were shown. In conclusion, our results provide experimental evidence for the potential benefits of BACE1 inhibition in AD. The treatment effectively repaired AD-like pathophysiology and brain circuit dysfunctions in an AD mouse model, representing the early disease phase. These findings are encouraging with regard to clinical primary prevention strategies being currently tested.

ZUSAMMENFASSUNG

Die Alzheimer-Demenz ist die häufigste Ursache für Demenz und stellt eine groβe Herausforderung für unsere alternde Gesellschaft dar. Die Hypothese der β-Amyloid-Kaskade beschreibt die Anhäufung des hochtoxischen Aβ als Auslöser einer Kaskade an pathologischen Ereignissen, welche zu Entzündungsprozessen, Synapsen-, Nervenzellverlust und zur Entstehung von Tau-Neurofibrillenbündel führt. Demnach stellt das Aβ-Peptid ein vielversprechendes therapeutisches Ziel dar. Durch die pharmakologische Hemmung des BACE1 Enzyms, welches an Aβs Bildung beteiligt ist, kann die übermäβige Anhäufung des Peptids im Gehirn verhindert werden. In der Tat konnten potente BACE1-Inhibitoren in präklinischen Studien zeigen, dass die zerebrale Aβ-Belastung in transgenen AD-Modellen wirksam reduziert wurde. Jedoch war es unklar, wie sich solch eine Behandlung auf neuronale Funktionen *in vivo* auswirken würde. Darüber hinaus gaben präklinische und klinische Daten Anlass zu Bedenken hinsichtlich BACE1-abhängiger Nebenwirkungen auf synaptische Funktionen und Kognition. Um herauszufinden wie sich eine chronische Behandlung mittels BACE1-Inhibitoren auf die Gehirnaktivität auswirkt, wurden *in vivo* Zwei-Photonen-Fluoreszenzmikroskopie mit Widefield-Bildgebung in einem APP23xPS45 Alzheimer Mausmodell kombiniert. Auf zellulärer Ebene wurde neuronale Aktivität und auf langreichweitiger Ebene die Ausbreitung langsamer Wellen beurteilt. Darüber hinaus wurde der kognitiven Status der Mäuse mit Hilfe eines Verhaltenstests bewertet. Wir konnten zeigen, dass eine pharmakologische BACE1 Hemmung in einem Alzheimer-Mausmodell, welches die frühe amyloidogene Phase der Alzheimer-Krankheit modelliert, Hirnaktivität auf verschienen Ebenen wiederherstellt. Tatsächlich behebt chronische BACE1 Hemmung neuronale Hyperaktivität, stellt die gestörte Koherenz langsamer Wellen, sowie Gedächtnisdefizite

wieder her. Besonders bemerkenswert ist, dass die Hirnaktivität in alten Tieren trotz der ausgedehnten Aβ-Pathologie wiederhergestellt werden konnte. Dies deutet darauf hin, dass trotz der zahlreichen Aβ-Plaques zu diesem Zeitpunkt noch keine irreversible Neurodegeneration stattfand. Der Effekt der funktionellen Erholung der Gehirnschaltkreise konnte auf die Abnahme toxischer präfibrillärer Aβ-Spezies zurückgeführt werden, welche neuritischen Aβ-Plaques umgeben. Nach erfolgreicher Therapie und Wiederherstellung physiologischer Gehirnaktivität, löste die Wiedereinführung dieser löslichen Aβ-Spezies die beschriebenen zellulären und langreichweitigen Funktionsstörungen erneut aus. Hinsichtlich der Bedenken bezüglich BACE1-Inhibitor-abhängiger Nebenwirkungen auf zerebrale Aktivität und Kognition, konnte in den behandelten Tieren keine abnormalen Aktivitätsmuster oder andere Störungen des Phänotyps festgestellt werden. Zusammenfassend zeigen die experimentellen *in vivo* Ergebnisse einen potenziellen Nutzen der BACE1-Hemmung für die Behandlung der Alzheimer Pathologie. Die BACE1-Hemmung bewirkte eine effektive Reduzierung der zerebralen Aβ-Plaques und konnte zerebrale Funktionsstörungen in unserem AD-Mausmodell, welches die frühe Krankheitsphase repräsentiert, beheben. Diese Ergebnisse sind ermutigend im Hinblick auf klinische Primärpräventionsstrategien, welche derzeit getestet werden.

ABBREVIATIONS

AD	Alzheimer's disease
Aβ	Amyloid-beta
(a)CSF	(Artificial) cerebrospinal fluid
AICD	APP intracellular domain
APP	Amyloid precursor protein
ApoE	Apolipoprotein E
BACE1	Beta-site APP cleaving enzyme 1
Ca^{2+}	Calcium
CatD	Cathepsin-D
CHL1	Close homolog of L1
CNS	Central nervous system
CTF	C-terminal fragment
DMN	Default mode network
E/I	Excitation and inhibition
ErbB4	Receptor tyrosine-protein kinase
FA	Formic acid
Jag1	Jagged Canonical Notch Ligand 1
LTP	Long-term potentiation
MCI	Mild cognitive impairment
NaVβ2	Voltage-gated sodium channels beta 2
NfL	Neurofilament light chain protein
NMDA	N-methyl-D-aspartate receptor
NRG1	Neuregulin-1

OGB-1 AM	Oregon Green 488 acetoxymethyl ester
PET	Positron emission tomography
PiB	Pittsburgh compound B
PS1/2	Presenilin 1/2
ROI	Region of interest
sAPP-α	Soluble peptide APPα
sAPP-β	Soluble peptide APPβ
sAPP-η	Soluble peptide APPη
Sez6	Seizure-related gene 6
Sez6L	Seizure 6-like protein
TX	Triton X
WT	Wild-type

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1. INTRODUCTION

1.1. Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia in the elderly (60-70% of cases) (Scheltens et al. 2021). With the increase of aging societies, the prevalence is anticipated to triple worldwide by 2050 (Prince et al. 2016). As long as no efficient treatment prevents, stops, or delays the disease's progression, the impact of AD on health care systems and society will be disastrous. Therefore, research into the causes, prevention, and ultimately the treatment of AD represents a primary unmet medical need (El-Hayek et al. 2019). AD is clinically characterized by a progressive impairment of memory and several other cognitive domains. Prominent symptoms include episodic and semantic memory loss, language impairments, visuospatial impairments, and functional disability with a decline in everyday activities performance (Ballard et al. 2011). In advanced disease stages, motor and neuropsychiatric symptoms including agitation, depression, apathy, delusions, hallucinations, and sleep impairment become clinically prominent (Lyketsos et al. 2011).

AD is a chronic neurodegenerative disease with two pathognomonic features, which Alois Alzheimer first described in 1907 (Alzheimer et al. 1995). Neuropathologically, the AD brain is characterized by extracellular Aβ plaques and intracellular tau-containing neurofibrillary tangles (Albert et al. 2011). Although the emergence of new biomarkers including advanced imaging techniques or cerebrospinal fluid (CSF) analysis facilitates AD diagnosis, a definitive neuropathological diagnosis can only be made post-mortem (Albert et al. 2011).

Due to a lack of disease-modifying drugs, current guidelines recommend symptomatic treatments aiming for cognitive, behavioral, and functional enhancement (Cummings 2021). One therapeutic approach relies on the cholinergic hypothesis in AD. Indeed, cholinergic neurons vanish in the basal forebrain, and the resulting cholinergic neurotransmission deficiency is associated with the decline in cognition seen in AD patients (Mufson et al. 2002; Terry and Buccafusco 2003; Mesulam et al. 2004). To therapeutically counteract the cholinergic deficits, cholinesterase inhibitors (donepezil, galantamine, and rivastigmine), which increase the synaptic acetylcholine levels, are employed for the treatment of mild to moderate AD dementia (Cummings 2021). Moreover, excessive glutamate levels in AD brains may contribute to cognitive and functional decline. Memantine is an NMDA receptor antagonist that has been shown to block the effects of excessive glutamate and is recommended in moderate to severe AD (Cummings 2021). In addition to therapeutically addressing the disease's symptoms, many putative disease-modifying therapies have emerged and failed over the past years (Long and Holtzman 2019). Aducanumab – a passive anti-Aβ vaccine – is currently the only medication that intervenes into the Aβ pathophysiology and is approved by the FDA in symptomatic AD. However, there is considerable controversy in the field about this approval and the effectiveness of the drug. (Selkoe 2019; Sevigny et al. 2016; Mullard 2021).

1.2. The amyloid cascade hypothesis or why Aβ is crucial in AD

To this day, the exact causes of AD are not fully understood. The widely accepted Aβ cascade hypothesis - proposed in 1992 by Hardy and Jiggins - states that Aβ accumulation is a central event leading to a pathologic cascade, ultimately resulting in the full manifestation of AD's pathology. The hypothesis describes Aβ accumulation triggering

a devastating cascade of processes encompassing plaque formation, microgliosis, astrogliosis, synapse dysfunction, neuronal dystrophy, tau tangle formation, and cell loss (Hardy and Allsop 1991; Selkoe 1991; Hardy and Higgins 1992; Hardy and Selkoe 2002). The theory combined AD's histopathologic evidence with the finding that genetic mutations lead to a autosomal dominant AD form. Indeed, Aβ was found to be the main component of neuritic plaques in the AD brain tissue (Masters et al. 1985). Subsequently, researchers discovered genetic conditions linked to familial AD, in which the amount of Aβ secretion was increased, recognizing a link between them.

Understanding how genetic mutations impact Aβ's secretion is fundamental to understand how Aβ is generated (Figure 1). Aβ is released through the processing of the amyloid precursor protein (APP), which is an integral type 1 transmembrane glycoprotein that is strongly expressed in neurons (Tanzi et al. 1987; Goldgaber et al. 1987; Kang et al. 1987; Lichtenthaler, Haass, and Steiner 2011). The canonic APP proteolytic processing occurs through four different proteases: the α-secretase, β secretase, γ-secretase, and a recently discovered η secretase (O'Brien and Wong 2011). APP is either processed in the "amyloidogenic" pathway, resulting in Aβ secretion, the competing "non-amyloidogenic" pathway, or in a newly discovered alternative pathway. Interestingly, the rate of APP's proteolytic processing is neuronal activity-dependent (Nitsch et al. 1992; Kamenetz et al. 2003). The amyloidogenic pathway leads to Aβ excision from APP. First, the β-secretase BACE1 ("beta-site APP cleaving enzyme 1") - a member of the aspartyl protease family - cleaves APP extracellularly. A C99 fragment remains in the membrane and the extracellular release of the soluble APPβ fragment (sAPP-β) results from this step. Second, the γ secretase cleaves the C99 fragment into Aβ. The Aβ peptide is secreted into the extracellular space, while the remaining part of the C99 fragment "APP intracellular

domain" (AICD) is released into the cytosol. The Aβ peptide varies in length, from 38 to 43 amino acids, and this is determined by the distinct cleavage sites for the γ-secretase. Aβ1-40 (40 amino acids long) and Aβ1-42 (42 amino acids long) are the most abundant forms (Klafki et al. 1996). The non-amyloidogenic pathway prevents the Aβ-peptide formation. The α-secretase cleaves APP in the Aβ-region and generates the soluble APPα (sAPPα) that is released extracellularly, as well as the C83 fragment which remains in the membrane. The γ-secretase cuts C83 and subsequently, p3 is secreted extracellularly, while AICD is released into the cytosol. Other alternative pathways were recently discovered, such as the η-secretase cleaving pathway (Willem et al. 2015). The η-secretase processes APP to soluble sAPPη and the membrane-bound C-terminal fragment-η (CTF-η). The CTF-η is subsequently processed by either the α-secretase or β-secretase to generate the soluble, non-aggregating Aη-α or Aη-β fragments. After Aβ monomers have been secreted via the non-amyloidogenic pathway, they aggregate to dimers, oligomers, and protofibrils, defined as insoluble Aβ species. Those aggregate further to fibrils and plaques, which are part of the insoluble Aβ species (Selkoe 2008).

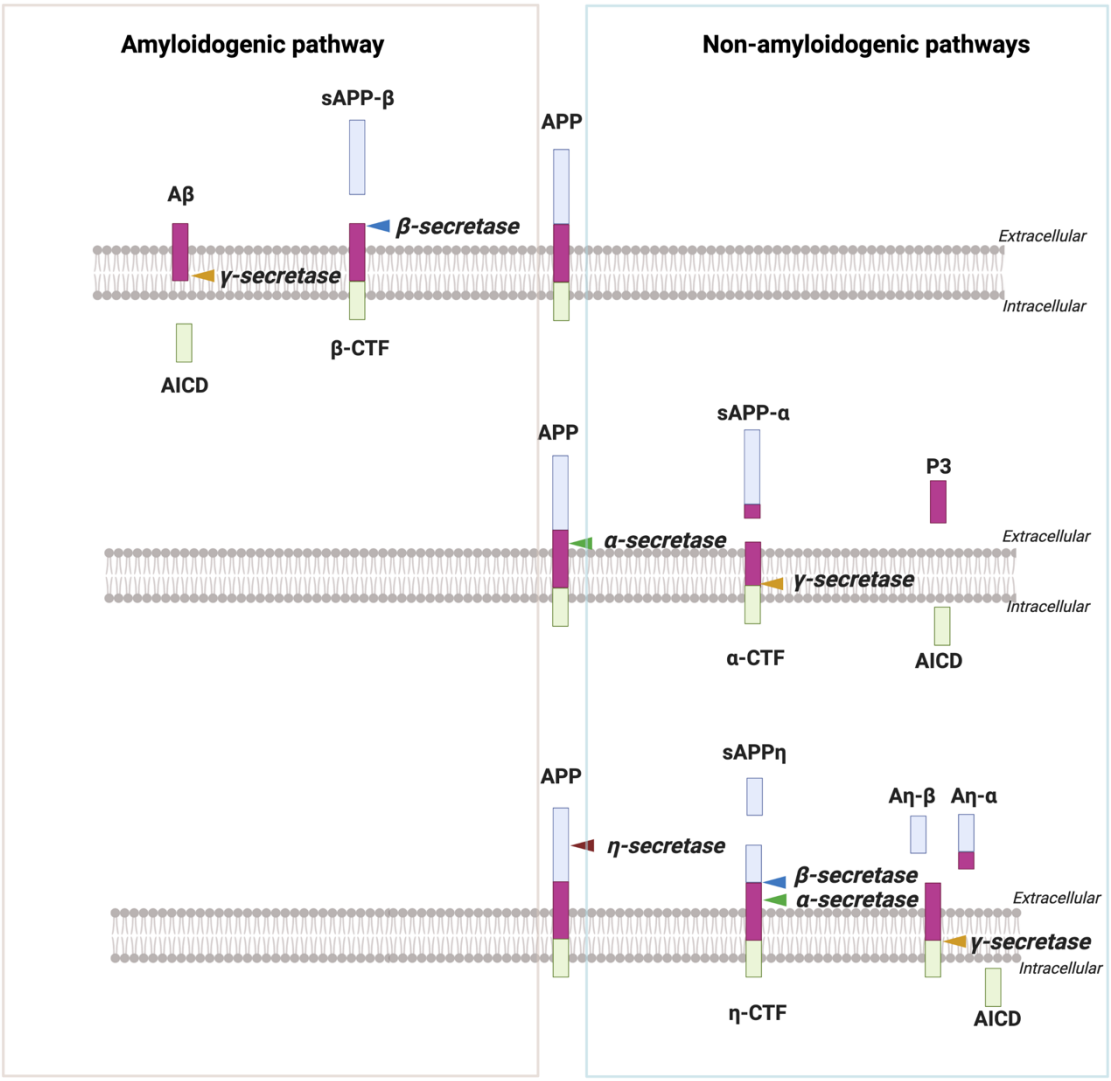

Figure 1: APP processing pathways and cleavage products.

(Top) *the amyloidogenic pathway is illustrated with APP being processed by the β-secretase and the γ-secretase,* ***(Middle)*** *the non-amyloidogenic pathway is illustrated with APP being processed by the α-secretase and the γ-secretase,* ***(Bottom)*** *the alternative pathway is illustrated with APP being processed by the η-secretase and the β-secretase or α-secretase.*

Compelling evidence supports the amyloid cascade hypothesis as genetic analysis of Down's syndrome and familial AD show that either the APP-, β-secretase- or the γ-secretase-coding genes are affected. For instance, Down's syndrome often leads to early AD. It has been found that the APP encoding gene is located on chromosome 21 (Goldgaber et al. 1987; Robakis et al. 1987; Tanzi et al. 1987; Kang et al. 1987). Therefore, due to an

additional APP copy and the resulting Aβ overproduction, individuals with Downs syndrome almost certainly display neuritic Aβ plaques by age 40 and are at great risk of developing AD (Lott and Head 2001; Wisniewski et al. 1985; Janicki and Dalton 2000; Johannsen, Christensen, and Mai 1996; Lai and Williams 1989; Oliver et al. 1998). Interestingly, a prospective longitudinal follow-up study of dementia in Down's syndrome individuals hinted at a possible causality between Aβ pathology and AD as Aβ strongly accumulated prior to the developed symptoms (Lai and Williams 1989). More evidence supporting the amyloid cascade hypothesis came from discovering dominantly inherited mutations causing familial AD. Mutations in presenilin 1 or 2 (PS1/PS2) are the most common cause of familial AD, and they have been shown to alter the γ-secretase's cleavage site on APP (Scheuner et al. 1996). As stated before, depending on where the γ-secretase cuts APP, Aβ1-40 and Aβ1-42 are mainly secreted. Indeed, the longer Aβ1-42 peptide is more prone to aggregate to oligomers than the shorter Aβ1-42 (Levy-Lahad et al. 1995; Scheuner et al. 1996; Citron et al. 1997; Borchelt et al. 1996; Duff et al. 1996; Wolfe et al. 1999; De Strooper et al. 1998). As described earlier, after the formation of oligomers, Aβ further assembles to protofibrils and fibrils that clump into sticky Aβ plaques that are a hallmark in the brains of AD patients (Haass and Selkoe 2007). Therefore, PS mutations that cause a surplus of longer Aβ1-42 species have been linked to AD (Scheuner et al. 1996; Bitan et al. 2003). Inversely the inactivation of PS 1/2 in mice completely prevented Aβ generation (Herreman et al. 2000). Dominantly inherited APP mutations modulate the affinity of APP to BACE1 and, therefore, the likelihood of being processed, resulting in increased amounts of released Aβ (Hampel et al. 2021). For instance, the Swedish mutation leads to increased β-secretase cleavage of mutated APP, resulting in enhanced Aβ peptide production and aggregation (Citron et al. 1992). Similarly, the APP variant A673V, discovered in an Italian family, is more

prone to amyloidogenic processing (Di Fede et al. 2009). In contrast, the Icelandic APP variant represents a protective mutation (Jonsson et al. 2012). The mutation renders APP less favorable for β-secretase cleavage, reducing Aβ production, and delaying the onset of AD. In sum, these observations provide a proof of principle that the β-secretase cleavage of APP plays a major role in the development of the Aβ pathology and AD.

The amyloid hypothesis encompasses increased Aβ production or decreased Aβ clearance resulting in an Aβ abundance and accumulation. The overwhelming majority of AD cases (~95%) occur sporadically and is associated with environmental or genetic risk factors. Genetic risk factors including ApoE ε4, Trem2, CR1, CD33, PICALM variants have been shown to impair Aβ clearance through, among other things, microglia dysfunction, resulting in increased cerebral Aβ loads (Karch and Goate 2015).

The amyloid cascade hypothesis proposes that Aβ accumulation is the primary event that initiates a cascade of pathologies resulting in AD (Selkoe 1991; Hardy and Higgins 1992). To investigate in which chronological order the pathologies occur in AD, longitudinal imaging studies involving familial AD mutations carrying families have confirmed the sequence of events. It also allowed to posit an AD pathology progression scheme (Jack et al. 2013; Jack and Holtzman 2013). Importantly, clinical biomarker studies show that CSF Aβ42 levels decline as early as 25 years before symptom onset, followed by cerebral Aβ accumulation measured by Pittsburgh compound B positron emission tomography scans (PiB-PET). Subsequently, increased CSF tau levels, cerebral glucose hypometabolism measured by radiolabeled fluorodeoxyglucose (FDG-) PET, progressive brain atrophy along with cognitive symptoms occur (Moscoso et al. 2021; Mielke et al. 2018; Janelidze et al. 2020; Timmers et al. 2019; Sperling et al. 2019; Jacobs et al. 2018; Pontecorvo et al. 2017; Hanseeuw et al. 2019; Bateman et al. 2012; Musiek and Holtzman 2012). Studies in

sporadic AD confirm the hypothesis, displaying similar dynamics (Villemagne et al. 2013). Figure 2 from (Selkoe and Hardy 2016) summarizes those biomarker changes.

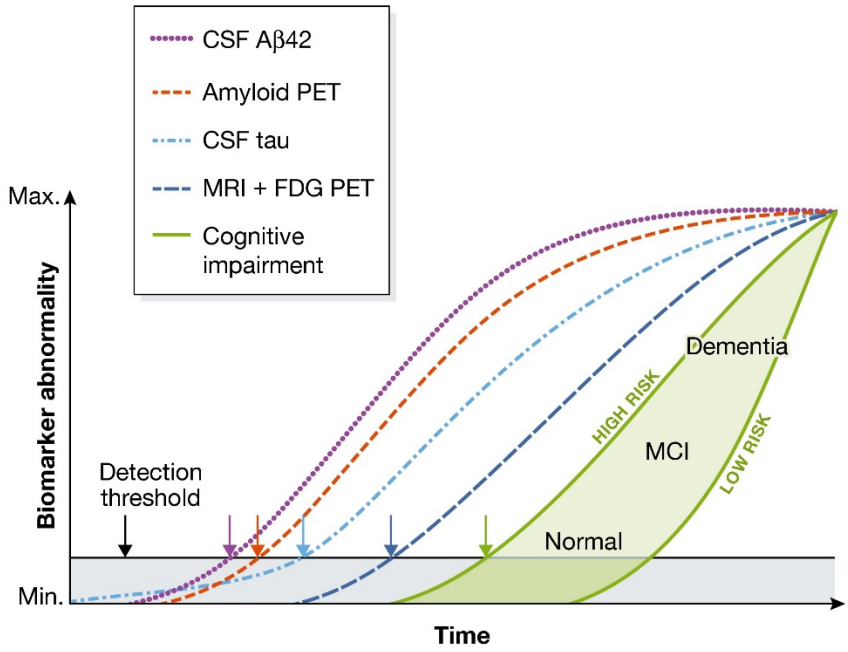

Figure 2: A temporal model for AD biomarker and clinical progression. Figure from (Selkoe and Hardy 2016).

The black horizontal line denotes the detection threshold, and the gray area represents the zone in which abnormal pathophysiological changes cannot be detected. Aβ deposition increases and arrows mark the point above the detection threshold. Tau pathology can precede Aβ deposition, but Aβ increase induces the acceleration of tauopathy with a rise in CSF tau above the detection threshold. FDG-PET and MRI changes follow. Cognitive impairment follows. CSF Aβ1-42 change represents a decrease in CSF Aβ1-42 levels.

1.3. Brain circuit dysfunctions in AD mouse models

In line with the amyloid cascade hypothesis, a significant amount of research focused on Aβ, which is believed to be the earliest pathology driver. To do so, AD mouse models were genetically engineered to enable basic AD research and investigate mechanisms underlying AD pathology.

1.3.1. Synaptic dysfunctions in AD models

Twenty years ago, a prominent theory stating that AD and its cognitive symptoms are based on " synaptic failure", was introduced (Selkoe 2002). It was based on two arguments: first, Aβ oligomers have been shown to induce synapse loss (Hong et al. 2016; Shankar et al. 2007; Selkoe 2002) and second, to disrupt synaptic function (Walsh et al. 2002; Barry et al. 2011; Klyubin et al. 2008; Shankar et al. 2008).

According to the hypothesis that synaptic activity is the basis for memory and cognition, cognitive decline in AD patients correlates most strongly with synapse loss than other AD pathologies (Terry, Masliah et al. 1991, DeKosky, Scheff et al. 1996). In fact, synapse loss occurs before plaque formation and independently of neurodegeneration. Synapse loss correlates strongly with soluble Aβ in the earliest stages of pathology (Hsia, Masliah et al. 1999, Lue, Kuo et al. 1999, D'Amelio, Cavallucci et al. 2011, Hong, Beja-Glasser et al. 2016). At later stages, dendritic spinous process loss is greatest near plaques (Spires, Meyer-Luehmann et al. 2005, Koffie, Meyer-Luehmann et al. 2009). The effect of Aβ oligomers on synapses has been confirmed in WT (wild-type) mice, as it triggered synapse loss in the healthy brain (Hong et al. 2016; Shankar et al. 2007). Initial insights into the influence of Aβ on synaptic function have been gained by *in vitro* studies (brain slices or cell cultures). Long-term potentiation (LTP) investigation is a widely accepted electrophysiological cellular correlate of synaptic function and plasticity based on an experimentally induced long-term amplification of synaptic transmission in the form of LTP and LTD (long term depression) (Bliss and Collingridge 1993; Lomo 2003). It has been shown that high Aβ load in brain slices via extrinsic application of Aβ in WT animals induces severe impairment of synaptic transmission (Hsia, Masliah et al. 1999, Selkoe 2002, Kamenetz, Tomita et al. 2003, Snyder, Nong et al. 2005, Hsieh, Boehm et al. 2006). Consistently, impaired LTP was observed in the hippocampus of AD mouse models with

intrinsic high Aβ loads (Chapman, White et al. 1999, Larson, Lynch et al. 1999), in WT mice intraventricularly injected with Aβ oligomers or in WT brain slices superfused with high levels of Aβ (Lambert, Barlow et al. 1998, Freir, Holscher et al. 2001, Walsh, Klyubin et al. 2002). Inversely, the selective reduction of Aβ oligomers resulted in the restoration of LTP (Walsh, Klyubin et al. 2002).

In sum, Aβ has been shown to directly impair synaptic morphology and function, which are on the basis of cognition and memory.

1.3.2. Cellular dysfunctions in AD models

The development of techniques such as two-photon calcium (Ca^{2+}) imaging and electrophysiological recordings enabled the assessment of neuronal activity with single-cell to cell population resolution in the living and intact brain tissue (Stosiek et al. 2003). The application of those techniques in transgenic mouse models bearing AD mutations made the functional investigation of cellular and synaptic mechanisms in the context of developing β-amyloidosis possible. Because the "synaptic failure" theory was very prominent and widely accepted, a global silencing of neurons was expected to be measured. As expected, Aβ depositing transgenic mice showed a massive disruption of cellular activity in the cerebral cortex, but surprisingly in the form of neuronal hyperactivity (Zott et al. 2019; Zott et al. 2018; Busche and Konnerth 2016; Busche et al. 2012; Busche et al. 2008; Grienberger et al. 2012; Siskova et al. 2014; Rudinskiy et al. 2012; Scala et al. 2015; Xu et al. 2015; Korzhova et al. 2021). Quantitative analysis in the APP23xPS45 AD mouse model showed strong impairments of neuronal activity. Indeed, 50% of neurons in cortical layer 2/3 displayed disrupted activity, with 21% of all neurons being hyperactive compared to WT control mice (Busche et al. 2008). In addition, hyperactive cells were located in the

vicinity of Aβ plaques (less than 60 µm radius). In the same mouse model, other regions such as the visual cortex (Grienberger et al. 2012) and the CA1 region of the hippocampus (Busche et al. 2012) also showed increased numbers of hyperactive cells. Further studies validated dysfunctional cell activity in Aβ depositing mouse models using imaging or electrophysiological measurements (Zott et al. 2019; Zott et al. 2018; Busche and Konnerth 2016; Busche et al. 2012; Busche et al. 2008; Grienberger et al. 2012; Siskova et al. 2014; Rudinskiy et al. 2012; Scala et al. 2015; Xu et al. 2015; Korzhova et al. 2021). Experiments in awake transgenic animals confirmed the observed cellular hyperactivity in anesthetized mice (Liebscher et al. 2016; Korzhova et al. 2021). These results clearly showed that neuronal activity the Aβ in depositing brain is impaired *in vivo*.

When considering the mechanism of neuronal hyperactivity in AD, evidence shows that hyperactivity results from a synaptic excitation and inhibition (E/I) imbalance. On the one hand, recent work from our laboratory showed that Aβ suppresses astrocytic glutamate reuptake at the synaptic cleft, which accumulates and results in increased postsynaptic excitation and neuronal hyperactivity (Zott et al. 2019). This study did not find evidence that GABAergic inhibition contributes strongly to Aβ-induced neuronal hyperactivity. Indeed, the hyperactivity-inducing effect of Aβ was canceled when glutamatergic antagonists D-APV and CNQX were applied in the CSF. In turn, earlier studies suggested that reduced GABAergic inhibition impair neuronal excitation and inhibition balance with a stronger excitation resulting in neuronal hyperactivity (Verret et al. 2012; Busche et al. 2008; Schmid et al. 2016; Sun et al. 2012; Palop and Mucke 2016; Martinez-Losa et al. 2018). Pharmacologically enhancing GABAergic neurotransmission was shown to rescue excess firing of cells (Busche et al. 2008), and therapeutic GABAergic enhancement improved cognitive outcomes in young AD mice (Sun et al. 2012). The

function of parvalbumin-positive interneurons, widely responsible for feedforward inhibition, was shown to be impaired (Viana da Silva et al. 2019), and somatostatin-positive interneurons involved in feedback inhibition were shown to be dysfunctional in AD models (Schmid et al. 2016). Interestingly, another study showed that Nav1.1 channels in parvalbumin-positive GABAergic neurons are impaired (Martinez-Losa et al. 2018; Verret et al. 2012) and restoring Nav1.1 levels recovered neuronal function, improved cognitive deficits, rescued the seizure phenotype and premature mortality (Verret et al. 2012). Moreover, neurons near amyloid plaques were shown to display less perisomatic GABAergic terminals and thus is possibly contributing to the observed E/I imbalance (Garcia-Marin et al. 2009).

Clinical observations and imaging studies show that cerebral network activity is abnormally increased at the early stages of AD (Zott et al. 2018). Indeed, an increased incidence of epileptic seizures and subclinical epileptiform activity has been reported in AD patients, suggesting network hyperexcitability and hyperactivity (Palop and Mucke 2009, Vossel, Beagle et al. 2013, Cretin, Sellal et al. 2016, Sarkis, Willment et al. 2017, Vossel, Tartaglia et al. 2017). The incidence of epileptic seizures during the course of AD has been estimated by various papers and displays a great variability (1.5-64%). However, most papers report 25% of epilepsy incidence and 50% incidence of epileptiform activity in AD patients (Friedman, Honig et al. 2012, Sherzai, Losey et al. 2014). The incidence is even higher in familial inherited AD forms (Shea, Chu et al. 2016). Functional magnetic resonance imaging (fMRI) studies enabled to measure the blood oxygen level-dependent BOLD signal, which reflects changes in the brain network activity in patients (Ogawa et al. 1990). Similar to our AD model, hyperactivity was observed in memory-relevant areas such as the hippocampus in early and presymptomatic AD (Sperling et al. 2009; Dickerson et al. 2005).

Even individuals at great risk for AD, such as asymptomatic familial AD subjects (Quiroz et al. 2010) and APOE-ε4 carriers (Bookheimer et al. 2000), displayed hippocampal hyperactivity. Importantly, the above-described hyperactivity was spatially associated with Aβ accumulations (Leal et al. 2017; Sperling et al. 2009). Other functional impairments in AD were found in the default-mode network (DMN), a large-scale brain network active under resting conditions such as daydreaming, and deactivates during goal-oriented tasks (Raichle et al. 2001; Buckner, Andrews-Hanna, and Schacter 2008). In AD, the deactivation of the DMN during cognitive exercises is strongly disturbed and the DMN is continuously active (Lustig et al. 2003; Buckner, Andrews-Hanna, and Schacter 2008; Sperling et al. 2009; Dickerson et al. 2005). Also, abnormally DMN activation has been observed in asymptomatic individuals with elevated Aβ levels or APOE ε4 risk allele-bearing individuals at risk for AD (Lustig et al. 2003; Persson et al. 2008; Sperling et al. 2009; Pihlajamaki et al. 2010).

It has been previously suggested that the observed hyperactivity could be a compensatory mechanism for a simultaneous silencing of cells. This possibility is rather unlikely as two main arguments suggest that neuronal hyperactivity drives the pathophysiological disease progression. First, excess neuronal activity has been shown to impair the coding accuracy of local neuronal networks. Indeed, the sensory integration of neurons and their coding accuracy is disrupted (Cacucci et al. 2008; Galloway et al. 2018; Koh et al. 2010; Liebscher et al. 2016; Mably et al. 2017; Wilson et al. 2005; Grienberger et al. 2012). For instance, specific neurons of the visual cortex, responsive to a particular visual stimulus, lost their ability to selectively respond to their specific visual stimulus in transgenic AD model mice (Grienberger et al. 2012; Rudinskiy et al. 2012). The local circuit disruption directly impacted cognition in mice , as they performed worse in a visual pattern

discrimination task (Grienberger et al. 2012). In addition, several studies have shown that hippocampal "place cells" encoding spatial orientation were active in an uncontrolled and stimulus-unspecific manner in transgenic models and aged rats (Cacucci et al. 2008; Galloway et al. 2018; Koh et al. 2010; Mably et al. 2017; Wilson et al. 2005). Again, animals showed lower performance on a spatial memory tasks due to the hippocampal cell activity disruption. Conversely, therapeutic lowering of excess activity using antiepileptic drugs in aged rats (Koh et al. 2010) and AD transgenic mice improved spatial learning and memory (Sanchez et al. 2012). Also, AD models are more prone to epileptiform activity, seizures, and related premature mortality (Martinez-Losa et al. 2018; Palop et al. 2007; Palop and Mucke 2016; Verret et al. 2012; Minkeviciene et al. 2009). Second, hyperactivity triggers a vicious cycle of increased Aβ secretion. Indeed, neuronal hyperactivity has been shown to promote extracellular Aβ secretion and plaque deposition (Bero et al. 2011; Huijbers et al. 2015; Leal et al. 2017; Yamamoto et al. 2015; Yuan and Grutzendler 2016; Kastanenka et al. 2019). Indeed, activation of specific brain regions can lead to amyloid plaque deposition in those areas, and inhibition of neuronal activity leads to a decreased amyloid plaque load. Also, high neuronal activity alters the Aβ1-40/ Aβ1-42 balance, which was shown to promote Aβ's aggregation (Dolev et al. 2013). In line with evidence from mouse models, brain hyperactivation is rather dysfunctional than compensatory in the clinical setting. Regarding the pathology driving characteristic of neuronal hyperactivity, the level of regional brain activity in AD patients could predict the extent of Aβ accumulation in this region (Leal et al. 2017). The disabling effect of hyperactivity on cognition has also been demonstrated in patients. Indeed, the extend of hippocampal hyperactivity in AD patients was linked to the degree of present and subsequent clinical cognitive deficits (O'Brien et al. 2010; Miller et al. 2008). Interestingly, the antiepileptic compound levetiracetam

normalized hippocampal activity and improved cognition by therapeutically suppressing deleterious hyperactivation in individuals with mild cognitive impairment due to AD (Bakker et al. 2015; Bakker et al. 2012). Also, epileptic activity in AD patients was shown to be associated with an earlier onset of cognitive decline (Vossel et al. 2017). These preclinical and clinical results highlight that excessive neuronal activity is rather dysfunctional.

Bridging experimental evidence from cellular-level and brain-wide activity monitoring with fMRI and electroencephalography defines neuronal hyperactivity as a key feature of early-stage AD.

1.3.3. Long-range circuit dysfunction in AD models

Aβ accumulation was found to not only impair cerebral activity at the cellular level but also to break down long-range circuit activity in mouse models and patients. Slow-waves or slow oscillations occurring during non-REM (rapid eye movement) sleep are generated with a frequency below 1 Hz in the neocortex via Up- and Downstate transitions (Stroh et al. 2013). The depolarized Upstate travels through the whole brain and is followed by a hyperpolarized down-state. These correlated activations of vast brain areas, including the thalamus and the hippocampus, are believed to play a crucial role in transferring memory content from the short term storage in the hippocampus to the long-term memory in the neocortex (Steriade and Timofeev 2003; Rolls et al. 2011; Landsness et al. 2009; Diekelmann and Born 2010). The “active system consolidation theory” is build on this observation and suggests that encoded memories are periodically reactivated during sleep (Diekelmann and Born 2010; Rasch and Born 2013). Thus, memory consolidation requires coordinated activation of the hippocampus, thalamus, and neocortex. Widefield Ca^{2+}-

imaging in AD mouse models allowed to investigate the mechanism of slow-wave disruptions and long-range circuit coherence and demonstrated a complete desynchronization of brain wide slow-waves (Busche, Kekus, et al. 2015). Indeed, Aβ accumulations compromised the coordinated spreading of slow-waves, leading to a functional decoupling of brain areas involved in memory consolidation (neocortex, hippocampus, thalamus) (Castano-Prat et al. 2019; Busche, Kekus, et al. 2015; Kastanenka et al. 2017). Moreover, by introducing soluble Aβ to WT animals, the coherence of slow-waves activity was impaired (Busche, Kekus, et al. 2015). Interestingly, the long-range circuit disruption also resulted from excitation and inhibition imbalance, as the slow-waves coherence was restored by pharmacologically increasing the GABAergic neurotransmission (Busche, Kekus, et al. 2015; Kastanenka et al. 2017). The mechanism of whether the slow-waves impairment is a result of cellular hyperactivity or if Aβ directly disturbs slow-wave coherence remains unclear. As the function of slow-oscillations is crucial for memory formation and consolidation (Diekelmann and Born 2010; Rasch and Born 2013), aged APP23xPS45 mice with disturbed slow-waves also display learning and memory defects (Busche, Kekus, et al. 2015). Inversely, restoring the slow-wave coherence improved memory performance in a spatial cognition task in the mice (Busche, Kekus, et al. 2015). AD mouse models with an intrinsic high Aβ load or the Aβ injection into the hippocampus of WT mice, not only triggered synaptic functional defects, but also cognitive defects (Nalbantoglu et al. 1997; Stephan, Laroche, and Davis 2001). The Morris water maze task assesses visuospatial cognition and memory in rodents. Its completion involves a range of sophisticated cognitive processes, including the acquisition and spatial location of visual cues that are processed, consolidated, retained, and retrieved to locate a hidden platform to escape the water by the animal (Morris 1984).

Slow-wave disruption during NREM sleep and memory impairment is similarly described in AD patients and individuals at risk for AD (Westerberg et al. 2012; Hita-Yanez et al. 2012). A study combining Aβ-PET imaging, fMRI, EEG, and memory performance assessments, showed the direct association between strong Aβ accumulating regions, reduced slow-waves, and consequentially impaired overnight memory consolidation (Mander et al. 2015). Alterations of slow-waves during NREM sleep were related to sleep disruptions and worsened cognition observed in AD patients (Ju, Lucey, and Holtzman 2014). It is noteworthy that sleep disturbances are reported early in the course of AD and correlate with Aβ CSF levels and memory performance (Hita-Yanez et al. 2012; Westerberg et al. 2010; Ficca et al. 2000; Sprecher et al. 2017).

In sum, desynchronization of slow-waves and an impairment of long-range circuits have been reported in AD mouse models as well as in AD patients. They seem to bridge Aβ to memory loss, but the mechanism behind the impairment of slow-waves has yet to be elucidated.

1.4. BACE1 function in health and disease

1.4.1. Physiologic BACE1 function

The β-Site APP-cleaving enzyme 1 (BACE1) is a transmembrane aspartic protease first described in 1999 (Lin et al. 2000; Yan et al. 1999; Sinha et al. 1999; Hussain et al. 1999; Vassar et al. 1999). BACE1 is mainly expressed in the brain and the pancreatic tissue of mice and humans (Sinha et al. 1999; Vassar et al. 1999; Marcinkiewicz and Seidah 2000). Its activity and mRNA expression are higher in neurons than in resting glial cells and is particularly abundant at the presynaptic terminals (Haniu et al. 2000; Vassar et al. 1999;

Zhao et al. 2007; Zhu, Xiang, et al. 2018; Zhao et al. 1996). At the subcellular level, BACE1 activity is highest in the acidic secretory pathway compartments and concentrated in the Golgi apparatus and endosomes (Vassar et al. 1999). BACE1 is concentrated at presynaptic terminals of healthy and transgenic AD mouse models (Sadleir et al. 2016; Kandalepas et al. 2013). Its activity is particularly important for the physiologic central nervous system (CNS) development, and levels are highest in the early postnatal phase (Willem et al. 2006). BACE1 bears diverse critical physiological functions in synaptic development and function. A knock-out of BACE1 enables to study those phenotypes, as it causes multiple more or less severe phenotypes (see table 1). Past studies identified many novel BACE1 substrates in addition to APP, which could explain the observed BACE1 knock-out phenotypes.

Table 1: Overview of described BACE1 knock-out phenotypes. Table adapted from Barao et al.,2016.

Phenotype	Substrate	References
Less exploratory	-	(Harrison et al. 2003)
Increased lethality	-	(Dominguez et al. 2005; Hu et al. 2010)
Hypomyelination	NRG1	(Hu et al. 2006; Willem et al. 2006; Luo et al. 2011)
Impaired spatial and working memory	-	(Laird et al. 2005; Kobayashi et al. 2008; Filser et al. 2015; Nash et al. 2020)
Impaired synaptic plasticity	NRG1, Sez6	(Laird et al. 2005; Kobayashi et al. 2008; Filser et al. 2015; Wang et al. 2008; Das et al. 2021; Wang et al. 2010)
Reduced spine density	NRG1, Sez6	(Savonenko et al. 2008; Gunnersen et al. 2007)
Impaired astrogenesis & neurogenesis	Jag1	(Hu et al. 2013)
Axonal targeting errors	CHL1	(Kim et al. 2018; Ou-Yang et al. 2018; Zhou et al. 2012; Kuhn et al. 2012; Hitt et al. 2012)
Impaired muscle spindles	NRG1	(Cheret et al. 2013)
Retinal pathology	VEGFR1	(Cai et al. 2012)
Schizophrenia-like phenotype	NRG1-erbB4	(Savonenko et al. 2008; Kobayashi et al. 2008)
Epileptic like seizures	NaVβ2, NRG1-erbB4	(Hitt et al. 2010; Hu et al. 2010; Kobayashi et al. 2008; Savonenko et al. 2008; Gerlai, Pisacane, and Erickson 2000; Stefansson et al. 2002)

The interaction of Neuregulin-1 and the Receptor tyrosine-protein kinase (NRG1-erbB4) plays a well-studied role in the regulation of axonal organization and myelination, the neuronal migration of glutamatergic and GABAergic neurons during early postnatal development, and synaptic plasticity (Willem, Lammich, and Haass 2009; Savonenko et al. 2008; Fleck et al. 2012; Hu et al. 2016; Hu et al. 2006; Willem et al. 2006; Michailov et al. 2004; Ou-Yang et al. 2018). BACE1 knock-out animals display a marked reduction of myelin sheath thickness in the sciatic nerves (Willem et al. 2015) and impaired axonal myelination (Luo et al. 2011). In addition, BACE1 knock-out mice show elevated pain sensitivity and impaired motor and sensory functions (Hu et al. 2006). Moreover, BACE1-NRG1 processing is required for the formation, maturation, and maintenance of muscle spindles, a proprioceptive organ critical for motor coordination (Cheret et al. 2013). Moreover, the lack of NRG1-erbB4 activation in BACE1 knock-out mice dysregulates synaptic E/I balance leading to impaired inhibitory cortical circuits (Unda, Kwan, and Singh 2016; Navarro-Gonzalez et al. 2021; Gu et al. 2016; Ting et al. 2011). NRG1-erbB4 activation strengthens the glutamatergic synapses (Li et al. 2007) and is required for the presynaptic formation of inhibitory synapses (Mei and Nave 2014). However, knocking out BACE1 results overall in excessive neuronal activation (Savonenko et al. 2008) and manifests in behavioral impairments such as novelty-induced hyperactivity and epileptic-like seizures (Hitt et al. 2010; Hu et al. 2010; Kobayashi et al. 2008; Savonenko et al. 2008; Gerlai, Pisacane, and Erickson 2000; Stefansson et al. 2002). BACE1 deficient mice also display synaptic activity and plasticity dysfunctions (Wang et al. 2010; Wang et al. 2008; Das et al. 2021). Indeed, at both the Schaffer collaterals and the mossy fiber projections in the hippocampus - the location where BACE1 is most expressed –presynaptic function is disrupted with impaired LTP (Wang et al. 2010; Wang et al. 2008; Laird et al. 2005; Kobayashi et al. 2008). A recent

study completes the picture of BACE1's role in synaptic functioning by displaying how the enzyme is involved in presynaptic vesicle release. BACE1 is required for optimal synaptic vesicle docking in the synaptic active zones (Das et al. 2021), possibly explaining why synaptic dysfunctions are reported in BACE1 knock-out mice.

Another crucial substrate of BACE1 is the voltage-gated sodium channels beta 2 (NaVβ2) - its BACE1-dependent processing controls the cell-surface-density of voltage-gated sodium channels and therefore modulates the neuronal excitability (Kim et al. 2007). Literature is not consistent about whether BACE1 inhibition increases or decreases the density of voltage-gated sodium channels (Hitt et al. 2012; Hitt et al. 2010). A study reported that BACE1 knock-out neurons show increased NaVβ2-density on the cellular surface, increased sodium currents, that they are hyperactive and thus provide an explanation for seizure susceptibility in these mice (Hu et al. 2010). Inversely, another study reported that cortical neurons of BACE1 knock-out mice display lower sodium ion current densities compared to WT neurons (Dominguez et al. 2005).

The seizure-related gene 6 (Sez6) and seizure 6-like protein (Sez6L) are BACE1 substrates that are important for the morphological basis of the synaptic functioning (Pigoni et al. 2016; Kuhn et al. 2012). In the absence of Sez-6, the dendritic morphology of cortical pyramidal neurons is impaired. The analyzed neurons display short dendrites, increased dendritic arborization, reduced synaptic density, functional LTP, and working memory impairment (Pigoni et al. 2020; Kim, Gunnersen, and Tan 2002; Nash et al. 2020; Gunnersen et al. 2007; Miyazaki et al. 2006; Zhu, Xiang, et al. 2018). Therefore, these data imply, that BACE1's proteolytic activity is essential for the formation and maturation of synapses.

BACE1 cleaves the close homolog of L1 (CLH1) substrate, which is required for axon guidance, cell adhesion, neuronal migration, and neurite outgrowth (Hitt et al. 2012; Zhou et al. 2012; Kim et al. 2018; Kuhn et al. 2012). A conditional knock-out in adult mice results in severe axon guidance and organization disruption in the mossy fiber pathway of the hippocampus. This result suggests that long-term BACE1 inhibition could disturb axonal structures and thus explain functional defects described (Ou-Yang et al. 2018).

Another BACE1 substrate, the Jagged-1 (Jag1) protein, is known to play a significant role in the CNS development (He et al. 2014). It regulates astrogenesis and neurogenesis via the Notch signaling pathway (Morrison et al. 2000; Gaiano and Fishell 2002; Hu et al. 2013). In BACE1 knock-out mice, the Jag1-Notch pathway is inactive so that increased astrogenesis and reduced neurogenesis are found (Hu et al. 2013; Wilhelmsson et al. 2012). The imbalance in total astrocytes and neurons might impact synaptic functions as the fine-tuned interplay of the tripartite synapse might be disrupted. Moreover, Jag1 and Notch are synaptic peptides, which are upregulated by synaptic activity and themselves contribute to synaptic plasticity and memory formation (Alberi et al. 2011). This could explain the observed cognitive deficits in Bace1 knock-out mice. BACE1 inhibitors do not directly impact the Notch pathway, contrarily to γ-secretase inhibitors, that displayed severe side effects in Phase III clinical trial most likely through Notch inhibition (Doody et al. 2013).

1.4.2. Pathophysiologic BACE1 functions in AD

The body of literature describes BACE1 as a decisive player and potential therapeutical target in AD. It accounts for most of the β-secretase activity in the amyloidogenic APP processing (Yan et al. 1999; Vassar et al. 1999). BACE1 levels and its enzymatic activity are twofold higher in the neocortex of AD patients than in healthy

subjects (Li et al. 2004; Yang et al. 2003; Holsinger et al. 2002; Johnston et al. 2005; Fukumoto et al. 2002). At the subcellular level, BACE1 mainly accumulates in normal and dystrophic presynaptic terminals surrounding Aβ plaques in AD brains mouse models and patients (Kandalepas et al. 2013; Zhao et al. 2007). It has been suggested that the BACE1 elevation is a pathological response to deposing Aβ and may drive a positive-feedback loop enhancing local Aβ generation and accelerating the AD pathogenesis (Kandalepas et al. 2013; Zhao et al. 2007; Sadleir et al. 2014; Sadleir et al. 2016; Zhang et al. 2009). Moreover, elevated BACE1 levels have been detected in the CSF of individuals with AD, raising the possibility of utilizing this finding as a diagnostical tool (Verheijen et al. 2006; Holsinger et al. 2004; Holsinger et al. 2006). Interestingly, sporadic AD risk factors such as traumatic brain injury, stroke, cardiovascular events increase the cerebral BACE1 expression (Walker et al. 2012). Studies show that cellular (Vassar et al., 2009) and even environmental stresses drive the BACE1 expression up to AD levels in mouse brains (Devi et al. 2010). Therefore, BACE1 seems to be a stress-response protein that increases in reaction to oxidative stress (Tamagno et al. 2002), hypoxia (Zhang et al. 2007b), ischemia (Wen et al. 2004), apoptosis (Tesco et al. 2007), traumatic brain injury (Blasko et al. 2004), and impaired glucose metabolism (Velliquette, O'Connor, and Vassar 2005). Thus, it might be possible that environmental and cellular stress that are sporadic AD risk factors trigger the pathological AD cascade by increasing BACE1 activity and therefore Aβ accumulation.

1.5. Preclinical BACE1 inhibitor development and the use of NB-360

As BACE1 is responsible for the first step of the amyloidogenic APP processing pathway, its (partial) inhibition represents a promising strategy for AD treatment. Diverse therapeutic BACE1 inhibitors and modulators have been developed since then. For this aim, *in vivo* pharmacological properties, such as oral bioavailability, long serum half-life, or blood-brain barrier-penetration, had to be considered. Therefore, BACE1 inhibitors must be large enough to display sufficient BACE1 affinity to block the enzymes' active site effectively, be small enough to ensure blood-brain-barrier penetration, and be lipophilic to reach the endosomal lumen, where the active BACE1 is located (Vassar 2014). Preclinical BACE1 inhibitors have successfully been developed and effectively reduced cerebral Aβ in animal models (Vassar et al. 2014).

This study used a potent and well-characterized BACE1 inhibitor from Novartis that showed great potential in reducing brain Aβ levels. NB-360 shows high oral bioavailability and an excellent blood-brain-barrier penetration (Neumann et al. 2015). Besides the NB-360 treatments' effect on Aβ accumulations and dynamics, more aspects of AD have been subject of interest. Neuroinflammation is a hallmark of AD, and Aβ pathology is thought to trigger a pro-inflammatory response that drives the pathology and can be tracked by microglia activation states (Leng and Edison 2021). Notably, activated microglia and astrocytes are mainly found in the vicinity of plaques (Stalder et al. 1999). The NB-360 treatment led to a reduction in the number of neuroinflammatory cells back to control levels (Neumann et al. 2015). Studies in AD mouse models showed that the animal's CSF analysis reflects the temporal sequence and magnitude of Aβ and Tau changes in the CSF of patients (Maia et al. 2013; Schelle et al. 2017). A chronic NB-360 therapy did not only

reduce the Aβ pathology but also had a suppressing effect on CSF tau levels, suggesting that preventing Aβ accumulation delays further pathologies of the Aβ cascade (Schelle et al. 2017). The neurofilament light chain protein (NfL) is a neural cytoskeleton protein part of synapses (Petzold 2005; Yuan et al. 2015). NfL is particularly elevated in blood plasma and the CSF when neurodegeneration occurs (Chatterjee et al. 2018; Zetterberg et al. 2016). In old transgenic mice, NfL was elevated in the CSF and the plasma and correlated with the brain Aβ pathology (Bacioglu et al. 2016). The NB-360 treatment significantly reduced NfL in the CSF but was insufficient to substantially reduce fractions in the plasma (Bacioglu et al. 2016). This data suggests that the NB-360 treatment may lower neurodegeneration through Aβ reduction. Besides extracellular plaques, amyloid angiopathy is part of the cerebral Aβ pathology. The latter presents a risk factor for vasogenic edema and cerebral microhemorrhages for anti-Aβ immunotherapies (Salloway et al. 2014; Sperling et al. 2012). This raises the need to monitor this severe reaction in anti-Aβ directed therapies. The NB-360 treatment in APP23 mice, which bear amyloid angiopathy (Klohs et al. 2014; Beckmann et al. 2011), did not worsen microhemorrhages compared to the control group suggesting that this side effect is not a major concern (Beckmann et al. 2016).

It seems essential to reassure concerns raised from the observed phenotypes in the BACE1 knock-out mice. The side effects seen in BACE1 knock-out mice are mainly related to developmental signaling pathways, thus less worrying for a potential therapeutical BACE1 inhibition in the adult age. For example, myelination, cellular migration, and differentiation are processes completed by the time of adulthood. However, other processes such as muscle spindle maintenance, axon guidance, and neurogenesis are ongoing processes and thus might be disrupted by chronic BACE1 inhibition (Barao et al.

2016). Another limitation is the limited selectivity of inhibitors and the off-target inhibitions of cathepsin-D (CatD), leading to ocular and liver toxicity (Zuhl et al. 2016; Cebers et al. 2016; May et al. 2015; May et al. 2011). Non-specific BACE1 inhibitors induce BACE2 inhibition and result in hair depigmentation (Shimshek et al. 2016). When considering BACE1 mechanism-based toxicities, one must consider the degree of pharmacological BACE1 inhibition. For instance, the 100% BACE1 inhibition seen in BACE1 knock-out mice is neither achieved nor necessary in therapeutical settings, thus decreasing the risk of BACE1 related side-effects.

2. AIM OF THE THESIS

This project aimed to investigate how BACE1 inhibition and subsequent Aβ reduction affects neuronal circuit activity in an AD mouse model. For this purpose, *in vivo* Ca^{2+}-imaging techniques were combined in the APP23xPS45 AD mouse model. The two-photon Ca^{2+}-imaging technique was employed to monitor brain activity on the cellular level, and one-photon wide-field Ca^{2+}-imaging revealed the brain's long-range circuit's function. Mice behavioral phenotypes were assessed to investigate the treatment effects on cognition. After monitoring the impact of treatment on neuronal circuit function and cognition, the next goal was to understand the molecular mechanisms behind the observed changes. Thus, biochemical Aβ quantification assays, immunohistochemistry, and pharmacological compound testing *in vivo* were performed.

3. METHODS

3.1. Study animals

I used female APP23xPS45 double-transgenic mice from six to eight months of age to study the effect of a BACE1 inhibitor treatment. These mice bear both the Swedish and the G384A-mutated PS1 (Mullan et al. 1992; Tanahashi et al. 1996) mutations. The Swedish double mutation has been identified in two families with early-onset Alzheimer´s disease from Sweden (Mullan et al. 1992). The base pair transversions guanine to thymine, adenine to cytosine, in the respective codons 670 and 671 characterize the Swedish double mutation. They cause the amino acid changes lysine to asparagine at residue 595 and methionine to leucine at position 596 in the translated APP. The transgenic mice bearing this mutation overproduce human mutant APP and display a strong increase of Aβ release in the CNS. PS1 is part of the γ-secretase, and the PS1 mutation leads to a relative rise in Aβ42 with the Aβ42/Aβ40 ratio shifting to higher Aβ42 levels, Aβ42 being more hydrophobic and prone to aggregate (Selkoe and Hardy 2016; Tanahashi et al. 1996). APP23xPS45 mice accumulate Aβ plaques in their brains starting at two months of age, and at the age of six months they display global Aβ pathology driven by Aβ overproduction (Busche, Grienberger, et al. 2015; Busche et al. 2012; Busche et al. 2008; Grienberger et al. 2012). Moreover, they exhibit neuronal dystrophy (Busche et al. 2008), astrocytic (Kuchibhotla et al. 2009; Verkhratsky et al. 2017; Rodriguez-Arellano et al. 2016), and microglia (Brawek et al. 2014) pathologies on the cellular level. On the phenotypic level, the transgenic mice display learning and memory deficits characterize the transgenic mice (Grienberger et al. 2012; Busche, Grienberger, et al. 2015; Busche et al. 2008). I used WT

littermates of the same age and sex for control experiments. The experiments were performed in conformity with well-fare regulations and were authorized by the state government of Bavaria, Germany.

3.2. BACE1 Inhibitor Treatment

The NB-360 treatment interferes with the amyloidogenic pathway and blocks the β-secretase BACE1. NB-360 is characterized by high oral bioavailability and is brain penetrable. I orally treated the transgenic animals chronically for six to eight weeks with vehicle or NB-360 containing food pellets (0.25 g/kg; Provimi Kliba, Kaiseraugst, Switzerland). The mice had continuous access to food until the *in vivo* imaging experiments, after which the animals were sacrificed. During the imaging experiments (approximately four hours), mice were anesthetized and had no food intake.

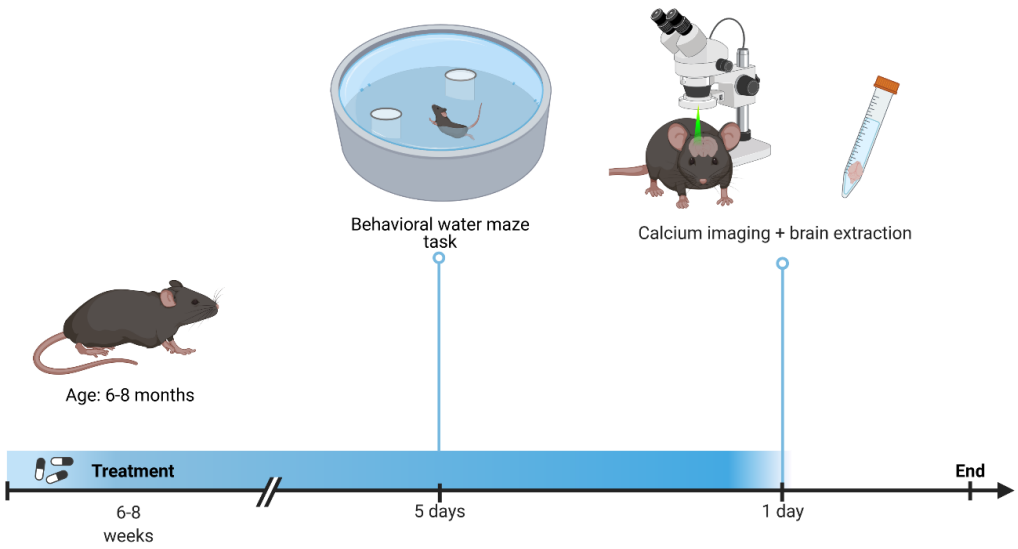

Figure 3: The timeline of the experimental assay.

Experimental timeline with treatment duration, behavioral task, and Ca^{2+}-imaging time points.

3.3. Behavioral testing

The transgenic mice were enrolled in a behavioral visual cue water maze task to test spatial memory performance. In this task, the mice had to swim in a circular water arena and discriminate between two visible platforms. One visible platform stayed in a fixed position, whereas the other changed locations from session to session. The incorrect platform sinks when the mouse climbs on it. Each day one session containing five trials per mouse was conducted. An additional session validated whether mice used spatial cues to locate the correct platform by moving it to the opposite quadrant in the water maze. If a wrong choice or an error of omission happened (when, the mouse could not reach a platform within 30 seconds – the trial was stopped), mice were put onto the correct platform for five seconds. Four training sessions preceded the fifth assessment day. The performance on day five was assessed by measuring the latency in seconds from the trial start to the reach of the right platform. The experimenter was blinded to the animal groups (WT, vehicle-treated transgenic, NB-360-treated transgenic) during the tasks. The behavioral data were analyzed using a linear mixed-effects model in MATLAB (Mathworks) using the fitlme -function. The fixed-effects factors are the assessment day and treatment (WT, vehicle transgenic, treated transgenic) with random-effects factors within one mouse.

3.4. *In Vivo* Ca^{2+}-imaging

In vivo two-photon imaging has many valuable advantages as it allows functional activity analysis of neurons in the intact mouse brain (Stosiek et al. 2003). This method allowed to investigate single cell to circuit functions in AD mouse models. I performed the Ca^{2+}-imaging experiments after behavioral testing. Surgical dissection was performed in

general anesthetized mice. The anesthesia was induced using the inhalation anesthetic isoflurane (1.5% vol/vol in 100% O2). The animal's vital signs were continuously monitored (heart rate, blood pressure, respiratory rate, temperature). The mouse's body temperature was kept constant at 37.5°C by placing the animal on a heating pad. Eye cream was applied to the mice's eyes to prevent ocular dryness. Subsequently, local anesthetics (50µl of a 2% xylocaine solution) and analgesics (metamizole, 200 mg/kg) were administered subcutaneously. To assess the depth of anesthesia, the flexor reflex and corneal reflex were tested. If the anesthesia was deep enough, the frontal skull (bregma 3 to 3.5mm, 0.5 to 1.5mm lateral to the midline (Paxinos and Franklin 2004) was exposed. Subsequently, the skull surface was air-dried, and a custom-made measurement chamber with a central opening was attached to the skull using dental cement. The skull was then thinned with a dental drill, and a one-mm-diameter craniotomy was cautiously performed to preserve the integrity of the brain tissue. The craniotomy was continuously perfused with artificial CSF (aCSF) (125mM NaCl, 4.5mM KCl, 26mM NaHCO3, 1.25mM NaH2PO4, 2mM CaCl2, 1mM MgCl2, 20mM glucose). The aCSF was gassed with a carbogen gas mixture (95% O2 and 5% CO2) to achieve a pH of 7.4 and heated to a physiological temperature of 37.5 °C. *In vivo* tissue staining of layer 2/3 neurons in the neocortex was performed with the Ca^{2+} sensitive fluorescent dye Oregon Green 488 BAPTA-1 AM (OGB-1 AM, Molecular Probes, Eugene, Oregon, USA). The absorption maximum of OGB-1 is at 488 nm, and the emission maximum is at 520 nm. Its high Ca^{2+} affinity (Kd=170nM) and signal robustness are advantageous for measurements in the experimental *in vivo* setting (Paredes et al. 2008). A 50-µg OGB-1 aliquot was dissolved in 20% Pluronic F-127 in DMSO solution and further diluted in a standard pipette solution (150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.4) to obtain a final concentration of 0.5 mM. The solution was filtered before injection (Millipore filter

with a pore size of 0.45.m) and loaded into a borosilicate glass micropipette (Hilgenberg GmbH, Malsfeld, Germany). The micropipette with an electrical resistance of 3 to 5 MΩ was guided to layer 2/3 (Manipulator LN-Mini 37 Methods Manipulator, Luigs & Neumann GmbH, Ratingen, Germany), and the dye was injected into the brain tissue by pressure application (10 psi, 550 mbar Picospritzer II, General Valve, Fairfield NJ, USA). After 60 minutes of incubation, I could see a spherically stained region 200-400µm in diameter (Stosiek et al. 2003). During imaging, a reduced isoflurane concentration (0.8% vol/vol in 100% O2) was used. *In vivo* Ca^{2+}-imaging was performed with a custom-built two-photon microscope (BX51WI, Olympus, Tokyo, Japan) coupled to a resonant galvomirror system (8 or 12 kHz, GSI) and controlled by scanning software (LabView (National Instruments), scanning the sample at an image acquisition frequency of 40Hz. The fluorescent dye was excited by a Ti: sapphire laser (Mai Tai, Spectra Physics, Mountain View, USA) with an 800 nm wavelength. The laser was focused through a 40x, 0.8 numerical aperture water immersion objective (Nikonenko et al.). Astrocytes could be differentiated from neurons by their typical morphology and high fluorescence due to their high resting Ca^{2+} state. The neuronal activity was recorded for more than five minutes in each focal plane. We used an average laser power of 15 mW, and special care was taken to avoid fluorescence bleaching of the stained region and cells. High-resolution overview images were obtained as projections of frames from different planes (1 frame per focal plane, step size one µm, one Hz acquisition rate).

After two-photon imaging of the single cells, the *in vivo* large-scale camera-based imaging was performed. For this purpose, the craniotomy was enlarged, and multiple cortex areas were stained with OGB-1. A blue light-emitting laser diode excited the fluorescence dye OGB-1 at 450 nm. Cortical slow-waves were recorded with a high-speed

digital camera (NeuroCCD; RedShirt Imaging) operating at 125 Hz (80 × 80 pixels) using a custom LabView data acquisition program (National Instruments).

3.5. Image analysis

Image analysis was performed offline using a custom-made LabView (National Instruments)-based image processing software. Manually selected regions of interest (ROI) were defined for neuronal somata, and single ROI-fluorescence was extracted from the raw image data in the form of Ca2+ fluorescence traces. Fluorescence and relative fluorescence (ΔF/F) changes within ROIs were plotted over a time axis. Ca^{2+} transients were detected in the Igor Pro software (Wavemetrics, Lake Oswego, Oregon, USA). Ca^{2+} signals were defined as relative fluorescence changes three times higher than the standard deviation of the baseline fluorescence noise band. We excluded astrocytes from the analysis based on their specific morphology. OGB-1 stains Aβ plaques to visualize them in the brain tissue (Garaschuk 2013). Our collaborators from the Institute of Computational Biology of the Helmholtz Center Munich supported us with the analysis of correlated Ca^{2+} transients. Ca^{2+}-traces were smoothed with a gaussian binominal filter with 1.7 seconds window size for the Ca^{2+} transient correlation analysis. We extracted the instantaneous phase of each trace by Hilbert transform and calculated the phase difference between these two traces. If the instantaneous phase difference was smaller than 54 degrees and the instantaneous phase of both traces was smaller than 72 degrees, then the sections were correlated. The correlated sections were selected, and the energy of both traces was calculated with the traces with minimum energy exceeding a threshold were preserved as correlated Ca^{2+} transients (minimum amplitude, 3× refNoise (mean of the absolute difference between raw and smoothed traces); minimum period, 0.7 s).

Recorded Ca^{2+}-slow oscillation signals were smoothed with a 50Hz low-pass filter. Next, the baseline fluorescence was calculated every 500 frames by defining the 10th percentile of the asymmetrical eight-second window. These calculated data points were assembled to constitute the fluorescence baseline. Then fluorescence traces were transformed into ΔF/F values (typical range, 0–0.1). Heart rate and breathing artifacts are in the frequency range of the relevant signal, which is why we avoided simple filtering. We instead used the denoising source separation algorithm, which identified the spatial pattern of the heart-rate artifact and projected it out of the data (de Cheveigne et al. 2013). To analyze the coherence of Ca^{2+} fluorescence signals, the cortical surface was segmented into four regions according to the occipital, somatosensory, motor, and frontal cortices. Then, the long-range spatial coherence of the Ca^{2+} fluorescence signals was examined by focusing on the frontal and occipital cortices signals. The signal coherence was assessed by the calculated correlation coefficients of 32 seconds traces of the Ca^{2+} signal in the cortical region.

3.6. Pharmacological experiments

NB-360-treated transgenic APP23xPS45 mice were imaged on the two-photon setup, and AβS26C cross-linked dimers (1 µM; JPT PeptideTechnologies) were washed in the aCSF and penetrated the brain topically. Neuronal activity was recorded before and 60 minutes after application. In another group of NB-360-treated transgenic APP23xPS45 mice imaged on the camera setup, Aβ1–40 and Aβ1–42 peptides at a concentration of 1 µM (Bachem) were washed in the aCSF. Slow-wave oscillations were recorded before (baseline) and ∼60 min after application. This group of experiments was performed by my colleague Dr. Zott, a Ph.D. student at our institute.

3.7. Aβ plaque quantification

After the experiment, the animal was decapitated, and coronal brain sections (30 µm) were obtained by vibratome (VT1000s; Leica). Histochemical staining with thioflavin-S (0.05% wt/vol) (T1892; Sigma-Aldrich) diluted 1:10,000 from 2% stock for 20 min (reference), and subsequent analysis enabled to quantify the Aβ -plaque burden. Pictures were taken using a light microscope connected to a camera (XC10; Olympus). ImageJ software was used to process the images and perform analysis of the thioflavin-S-stained cortex area. To quantify the prefibrillar amount of Aβ, sections were stained with an OC-antibody (AB2286; EMD Millipore) diluted 1:250 in PBS with 0.2% Triton X-100. The primary antibody was 4% goat serum to detect prefibrillar Aβ species, including oligomers. Sections were stained for two days at four degrees Celsius, washed, and then stained with an Alexa Fluor 647-conjugated secondary antibody (A21244; Thermo Fisher Scientific) diluted to 1:500 for 12 h. Images were obtained via confocal imaging using an Olympus FV3000 system, through a 60× oil-immersion objective (NA 1.42) at zoom 1, at a 1,024 × 1,024-pixel resolution with a three µm step size. Five random regions were chosen in each brain section, and two brain sections from each animal were selected. Acquired images were processed and analyzed the acquired images with ImageJ.

3.8. Biochemical Aβ quantification in animals.

Our collaborator from the Novartis Institutes for BioMedical Research (Dr. Ulf Neumann) performed biochemical Aβ level quantifications. Brains were homogenized in chilled TBS buffer (pH 7.4) with a Protease Inhibitor Cocktail (Roche Diagnostics) using a Branson Sonifier 450. The following protocol extracted soluble Aβ: to 50 µl homogenate 50

µl TBS or 50 µl 2% Triton X-100 (vol/vol) in TBS was added. The solution was then ultracentrifuged (100,000g) for 15 min, and the supernatant was analyzed. Insoluble Aβ could be extracted as follows: to 50 µl homogenate, 117 µl 100% formic acid was added, incubated for 15 min, and then neutralized. After centrifugation (20,300g), the supernatant was diluted and analyzed. Aβ quantification was performed by electrochemiluminescence immunoassay kit from Meso Scale Discovery using antibody 6E10.

3.9. Statistical analysis

Statistical hypothesis testing was performed using SPSS. The parametric test Welch's two-tailed t-test has been used to compare two independent groups. For comparing multiple groups, the multiple testing problem occurs. Therefore, a one-way analysis of variance (ANOVA) was performed first to see if the population means differ. If this was the case, I calculated which mean is different by the Tukey multiple comparison test. It compares the means pair by pair and adjusts for multiple testing. The t-test, ANOVA, and the Tukey multiple comparison tests assume that the data come from normally distributed populations with equal standard deviations in all groups.

4. RESULTS

4.1. The NB-360 effectively lowers Aβ loads in transgenic mice with advanced pathology

The effect of a BACE1 inhibitory treatment was investigated in 6-8 months APP23xPS45 transgenic AD mice by a six to eight weeks long treatment with the NB-360 BACE1 inhibitor or control vehicle food pellets. During chronic treatment, the non-selective NB-360 inhibitor did block the homolog BACE2. This resulted in previously reported depigmentation in some of the mice (Shimshek et al. 2016). No other visible phenotypic side-effects have been seen.

First, to confirm the effect of this inhibitor on cerebral Aβ pathologies (Neumann et al., 2015), I quantified the Aβ plaque load in the brain's mice collected after recordings were performed. Aβ plaque quantification via the histochemical thioflavin-S staining showed a drastic reduction of the Aβ plaque burden in the frontal cortex of treated mice (figure 4). The analysis of brain-homogenate-extracted Aβ comprised total Aβ in the formic acid (FA)-extracted fraction and soluble Aβ in the Triton-X–extracted fraction. Both insoluble and soluble Aβ 1–40 and Aβ 1–42 levels were significantly lowered in the forebrain of the NB-360 treated group (figure 4).

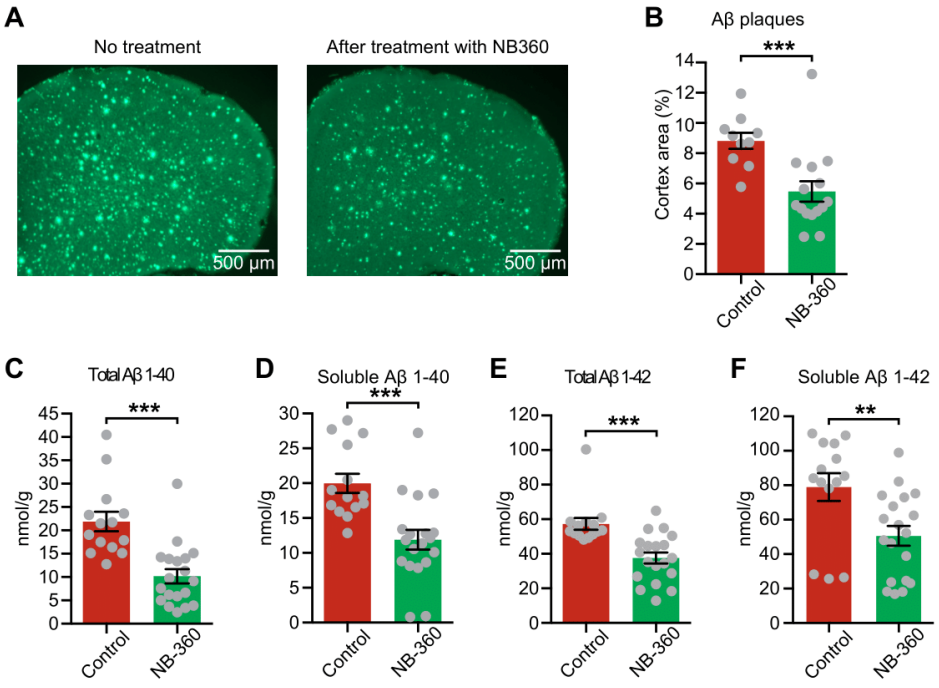

Figure 4: The NB-360 treatment clears the brain from the Aβ pathology. Figure modified from Keskin et al., 2017.

(A) *A representative thioflavin-S stained forebrain section of a vehicle-treated and an NB-360- treated APPxPS45 mouse. The overall amount of Aϐ plaques is reduced and plaques appear to be smaller.* ***(B)*** *Percentage of cortex area covered by thioflavin-S stained Aϐ plaques in the forebrain of vehicle- and NB-360-treated APP23xPS45 transgenic mice. Red bar represents untreated (n=10) and green bar treated (n=15) mice. The difference is highly significant (control: 8.83 + 0.54% vs. NB-360: 5.47 + 0.68%; two-sample t test, t = 3.84; df = 22.99; P = 0.0008).* ***(C-E)*** *Quantitative biochemical analysis shows total and soluble fractions of Aϐ 1-40 and Aϐ 1-42 in the forebrains.* ***(C)*** *Total Aϐ 1-40 fractions in forebrains of untreated (red bar; n = 14) and treated (green bar; n = 19) APP23xPS45 mice (FA-extracted: 21.89 + 2.10. nmol/g for control vs. 10.18 + 1.53 nmol/g for NB-360; two-sample t test, t = 4.52; df = 25.30; P= 0.0001).* ***(D)*** *Soluble Aϐ 1-40 (TX-extracted:19.98 + 1.39 pmol/g for control vs. 11.89 + 1.40 pmol/g for NB- 360; two-sample t test, t= 4.09; df = 30.27; P= 0.0003).* ***(E)*** *Fractions of total Aϐ 1-42 (FA-extracted: 57.31 + 3.47 nmol/g for control vs. 37.63 + 3.21 nmol/g for NB-360; two-sample test, £=4.17; df = 29.27; P = 0.0003).* ***(F)*** *Soluble Aϐ 1-42 (TX-extracted: 78.90 + 8.06 pmol/g for control vs. 50.64 + 5.70 pmol/g for NB-360; two-sample t test, t= 2.86; df = 24.79; P = 0.0084). Gray circles represent individual animals. **=P < 0.01, ***=P < 0.001. Error bars represent mean + SEM.*

4.2. The NB-360 treatment effectively repairs cellular dysfunctions

To test for the impact of BACE1 inhibition and Aβ reduction on neuronal functions, I first performed *in vivo* two-photon Ca^{2+}-imaging of large neuronal populations in the neocortical layer 2/3, in the forebrain of transgenic mice. I used an OGB-AM-dye to stain and visualize neurons and astrocytes. It was possible to differentiate astrocytes from neurons by their typical morphology and high fluorescence due to their high resting Ca^{2+} state. Therefore, I could monitor spontaneously occurring somatic Ca^{2+} transients in many individual neurons.

First, I did perform a multicell bolus loading in WT animals to confirm the WT-like cortical activity pattern (see chapter 3.4 for the technique description). Layer 2/3 neurons displayed Ca^{2+} transients at a 0.5 – 6 transients/minute frequency and showed the typical sparse firing pattern (figure 5). In this pattern, a single pyramidal neuron is infrequently active (Field 1987). Thus, displaying a frequency of 0.5-6 Ca^{2+} transients per minute were defined as “normally active.

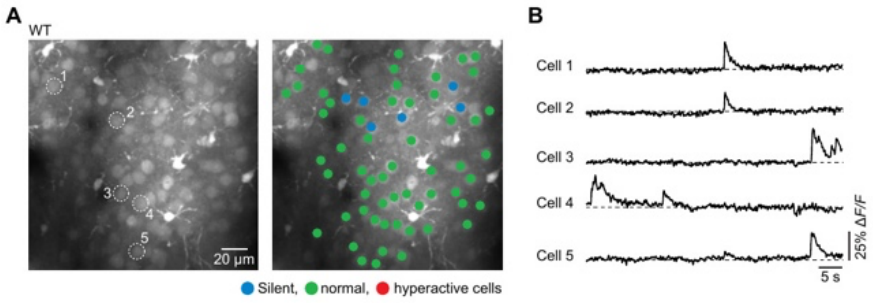

Figure 5: Neuronal activity monitored by Ca^{2+}-imaging in WT animals in vivo. Figure modified from Keskin et al., 2017.

(A) *Representative in vivo two-photon fluorescence images of cortical layer 2/3 neurons in the frontal cortex of WT mice and corresponding activity maps. Most cells display a normal Ca^{2+} transient frequency and are marked in green.* ***(B)*** *Ca^{2+} traces from five representative neurons marked in the image left.*

I imaged six-month-old APP23xPS45 mice that showed a high fraction of aberrant hyperactive neurons in layer 2/3, consistent with previous literature (Busche et al. 2008; Busche, Grienberger, et al. 2015; Grienberger et al. 2012). Those hyperactive cells displayed more than six transients per minute and were characterized by their spatial vicinity to Aβ plaques (figure 6). The quantification of the fraction of hyperactive cells revealed a highly significant increase of hyperactive cells compared to WT. The frequency distribution of all recorded neurons in APP23xPS45 was shifted towards higher frequencies than in WT mice. In summary, the number of hyperactive cells and the frequencies of single hyperactive cells were increased in Aβ plaque-bearing transgenic mice.

In APP23xPS45 mice treated with the BACE1 inhibitor NB-360, excessive activity was rescued and decreased to WT levels. The fraction of hyperactive neurons decreased compared to vehicle-treated controls and reached back to WT levels (figure 6). Indeed, frequency distributions of neuronal Ca^{2+} transients were shifted towards lower frequencies and resemble the WT's activity distribution. Interestingly, even though Aβ plaques were

still present in the brain parenchyma, the cells surrounding those presented a regular activity pattern (figure 6: plaques in yellow and normal active cells color-coded in green).

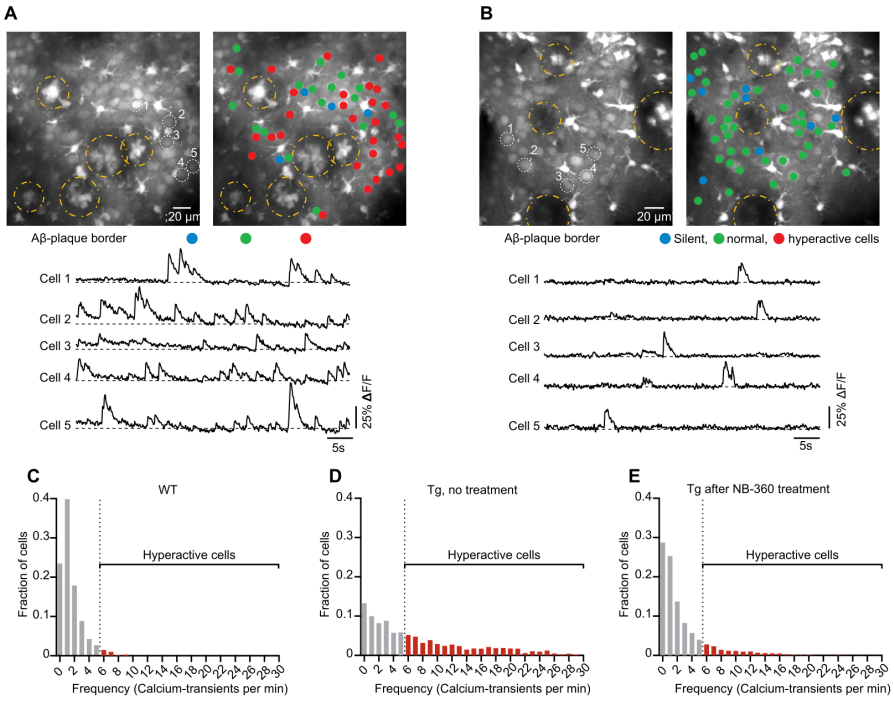

Figure 6: NB-360 rescues hyperactivity in transgenic APP23xPS45 mice. Figure modified from Keskin et al., 2017.

(A-B) *(Top)Representative in vivo two-photon fluorescence images of cortical layer 2/3 neurons in the frontal cortex of control APP23xPS45 (left) and NB-360 treated APP23xPS45 (roght) mice. Dashed yellow lines encircle the Aß plaques; neurons are color-coded with silent cells in blue, normally active cells in green, hyperactive cells in red. (Bottom) Five activity traces from representative neurons are labeled in the top image.* ***(C-E)*** *Histograms of frequency distribution.* ***(C)*** *Histogram of activity of neurons in WT (n= 1362 neurons in 5 mice).* ***(D)*** *The histogram shows a frequency shift to higher frequencies in APP23xPS45 (n = 1822 neurons in 8 mice).* ***(E)*** *The histogram shows a shift to lower frequencies and a substantial reduction of excessive cortical activity in NB360-treated APP23xPS45 (n = 2213 neurons in 10 mice). The limit frequency to hyperactivity is marked with a vertical dashed line at six transients/min.*

The extend of cellular activity repair depended on the duration of NB-360 treatment. A short-term (one week) treatment lowered the fraction of hyperactive cells to an intermediate level between the long-term and control groups (figure 7). The positive correlation between Aβ brain levels and the fraction of hyperactive neurons in individual

animals is worth mentioning. Interestingly, the higher the FA-extractable Aβ 1-40 fraction, the higher the fraction of the hyperactive cells (figure 7). The one-week NB-360 treatment partially reduced cerebral Aβ levels and thus partially rescued excessive hyperactivity in the cortex of transgenic mice.

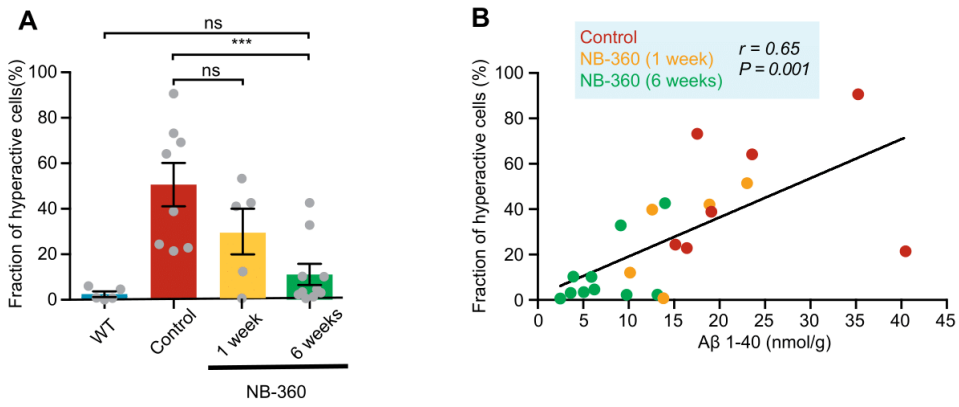

Figure 7: The reduction of hyperactive cell fractions is NB-360 dose-dependent and correlates with Aβ brain levels in APP23xPS45 mice. Figure modified from Keskin et al., 2017.

(A)*The fraction of hyperactive cells in WT (blue bar, n= 5) 2.48 + 1.20%, vehicle-treated (red bar, n= 8) 50.60 + 9.53%, one week NB-360-treated APP23xPS45 (yellow bar, n= 5): 29.22 ± 9.69%, six weeks NB-360-treated APP23xPS45 (green bar, n= 10) 11.15 ± 4.62%; F (2,20) = 13.66, P = 0.0002; Tukey's post hoc comparisons: WT vs. control P < 0.001, control vs NB-360 P < 0.001, WT vs. NB-360 P > 0.05. Circles represent individual animals. ***=P < 0.001, ns = P > 0.05. Error bars represent mean + SEM.* ***(B)*** *The hyperactive neuron fractions correlate positively with FA-extracted Aβ levels (n =22 mice). The circles represent individual mice, vehicle treatment (red), one week (yellow), and six weeks NB-360 treatment (green). Pearson's correlation coefficient R = 0.65, p = 0.001*

As described in the introduction, transgenic AD mice were described to display a higher incidence of epileptiform activity than WT mice (Martinez-Losa et al. 2018; Palop et al. 2007; Palop and Mucke 2016; Verret et al. 2012; Minkeviciene et al. 2009). Excessive active cells are more prone to a synchronous firing pattern, which may underlie epileptiform activity. To study this level of dysfunction, a pairwise correlation analysis per

recorded region was performed for all imaged neurons. As previously described, control transgenic mice presented abnormal synchronicity of neuronal activity compared to WT mice, displaying a typical sparse firing pattern (figure 5 and 8). The recordings and analyses showed that NB-360-treated transgenic mice displayed less neuronal activity synchronicity than control transgenic mice, and the level of activity correlation was similar to WT animals. Also, the degree of synchrony was positively correlated with the fraction of hyperactive cells in control and NB-360-treated APP23xPS45 mice.

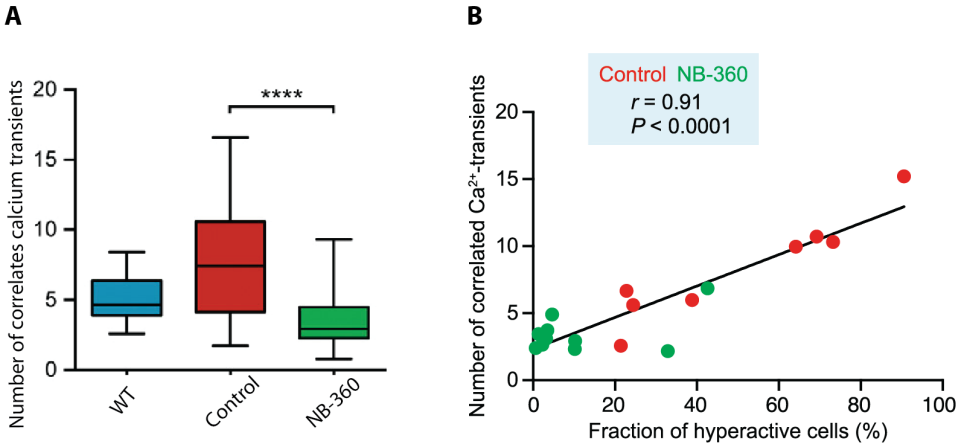

Figure 8: Hyperactive cells display correlated activity patterns. Figure modified from Keskin et al., 2017.

(A*) NB-360-treated transgenic mice (red bar, n = 64 regions in 8 mice) display less abnormal synchronicity of neurons compared to vehicle-treated transgenic mice (green bar, n = 81 regions in 10 mice), and a comparable level with WT (blue; n = 47 regions in five mice) [F(2,198) = 39.79, P=< 0.0001; Tukey's post hoc comparisons: P=< 0.0001 for WT vs. control and P=< 0.0001 for control vs. NB-360).****=P < 0.0001. (B) The synchronicity of transients is positively correlated with the fraction of hyperactive neurons in APP23xPS45 mice (n= 18 mice). The circles represent individual mice and color-coding represents vehicle treatment (red) and six weeks NB-360 treatment (green). ***=P < 0.001. Error bars represent mean + SEM. Pearson's correlation coefficient R = 0.91, p < 0.0001*

4.3. Potential side effects of NB-360 on brain circuits in WT

Next, I wanted to address potential unwanted BACE1 inhibitor-related side-effects on cerebral activity. To assess those, neuronal activity levels in NB-360-treated WT animals were monitored. There was no significant NB-360 treatment effect on WT activity levels. Indeed, the distribution of single-cell activity of NB-360-treated WT was similar to vehicle-treated WT mice (figure 9). Moreover, the fraction of hyperactive cells was not affected by the NB-360 treatment.

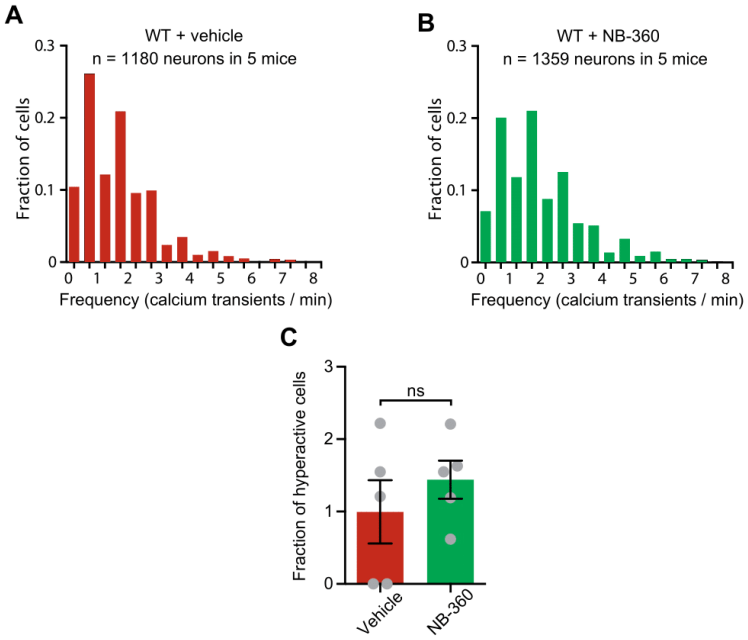

Figure 9: NB-360 does not change activity patterns in WT mice. Figure modified from Keskin et al., 2017.

(A, B) *Histogram of the frequency distribution of all neurons in vehicle-treated (n= 1180 neurons in 5 mice) and NB-360-treated (n= 1359 neurons in 5 mice) WT There is no change in activity patterns between vehicle-treated and NB-360-treated WT mice****.(C)*** *The fraction of hyperactive cells remains consistent in vehicle- (red bar, n=5 mice) and NB-360-treated (green bar, n=5 mice) WT mice.*

4.4. NB-360 treatment repairs long-range circuit dysfunction

Previous studies showed that Aβ accumulations in transgenic AD mice disrupt slow-wave oscillations of the neocortex (Castano-Prat et al. 2019; Busche, Kekus, et al. 2015; Kastanenka et al. 2017). I used wide-field Ca^{2+} fluorescence imaging of the cortical brain surface to monitor slow-wave oscillations in transgenic mice. Indeed, the long-range coherence of neuronal activity was massively impaired in our transgenic APP23xPS45 mouse model as described previously (figure 10)—this disruption presented in the form of brain-wide desynchronized and non-coherent slow-oscillations.

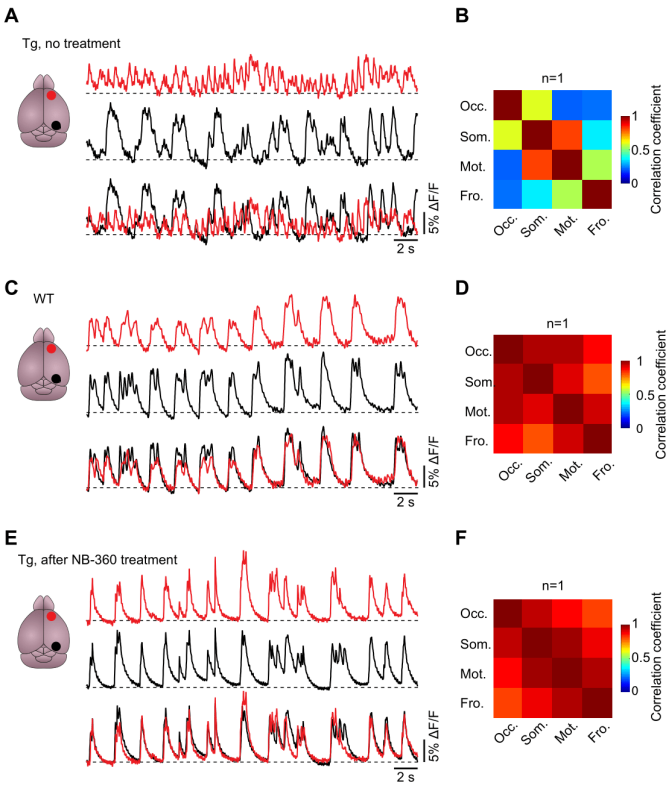

Figure 10: Long-range connectivity is recovered in NB-360-treated APP23xPS45. Figure modified from Keskin et al., 2017.

(A, C, E) *Long-range functional connectivity with example Ca^{2+} traces from the frontal (red), the occipital (black) cortices, and overlaid traces below. (A) Massive impairment of long-range coherence of slow-waves in an APP23xPS45 mouse.* ***(C)*** *Ca^{2+} traces from a WT mouse with coherent slow-wave oscillations. (E) Activity traces from an NB-360-treated APP23xPS45 mouse, showing that long-range coherence of slow-wave activity can be repaired.* ***(B, D, F)*** *Cross-correlation matrices of slow-wave oscillations between different cortices areas (occ. occipital, som. somatosensory, mot. motor, fro. frontal).*

Chronic NB-360 treatment did not only repair local single-cell activity but also restored the coherence in brain-wide long-range circuits. Three representative examples from each cohort (vehicle-treated transgenic, WT, NB-360-treated transgenic) made the impairment and restoration of slow-waves more evident (figure 10).

When further analyzing the slow-oscillations correlation in all animals, it became clear that the correlation coefficient between the occipital and frontal cortices was significantly improved by the NB-360 treatment (figure 11). Interestingly, there was a significant negative correlation between Aβ levels and the restoration of long-range coherence of slow-wave activity in individual animals. Therefore, the restoration of long-range activity correlated with Aβ level reduction.

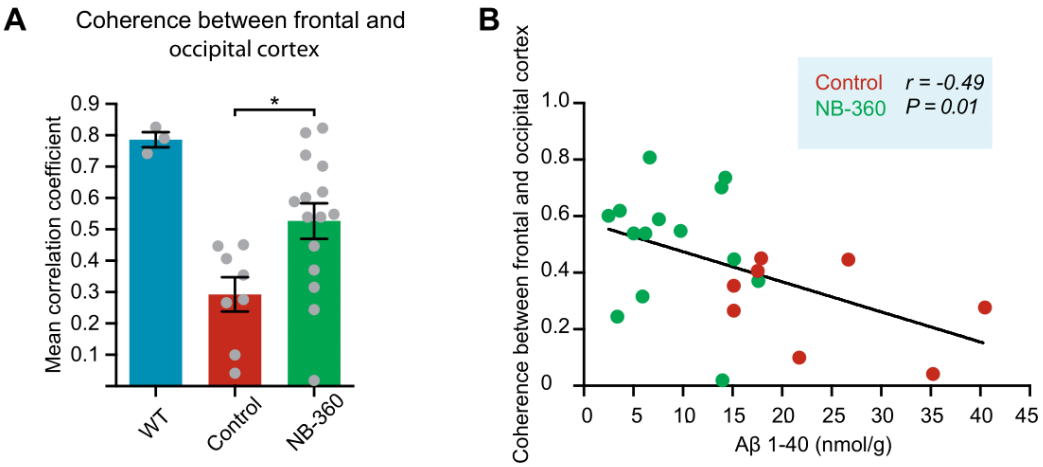

Figure 11: Correlation of slow-wave oscillations is restored in NB-360-treated APP23xPS45. Figure modified from Keskin et al., 2017.

(A) *The mean correlation coefficient between frontal and occipital cortices in transgenic (red bar; n = 8 mice), NB-360-treated transgenic (green bar;n = 15 mice), and WT (blue bar; n = 3 mice) mice. (WT: 0.79 ± 0.02 vs. control: 0.29 ± 0.05 vs. NB-360: 0.53 ±0.06; F (2,24) = 10.73, P = 0.0005; Tukey´s post hoc comparisons: P=< 0.001WT vs. control, P= < 0.05 control vs. NB-360, P= < 0.05 WT vs. NB-360).* ***(B)*** *Long-range circuit function negatively correlates with individual brain Aϐ levels (n = 22 mice, untreated APP23xPS45 in red, NB-360-treated APP23xPS45 in green. * = P < 0.05. Error bars represent mean ± SEM. Pearson's correlation coefficient R = -0.49, p = 0.01*

4.5. NB-360 repairs memory impairments

As slow-oscillations are known to be involved in learning and memory processes (Diekelmann, 2010 #275), I investigated the effects of slow-oscillations restoration on spatial learning and memory deficits in transgenic mice. For this purpose, mice were trained to perform a visual cue water maze task. Mice had to find a visible platform in a water maze task, and their escape latencies during training and on the assessment day (day 5) were recorded (figure 12). Untreated transgenic mice had longer escape latencies than WT mice in training sessions and at the assessment day. NB-360-treatment significantly improved the transgenic mice's cognitive performance so that they performed similar to the WT on the assessment day.

To exclude the possibility that prolonged escape latencies in transgenic vehicle-treated mice could be related to motor deficits, the swimming speed of all mice was investigated (figure 13). There was no difference in swimming speed between all mice. Thus, longer escape latencies were ascribed to longer trajectories in non-treated transgenic mice with cognitive deficits (figure 12: see the control group's trajectories compared to the treated group). Thus the NB-360 treatment rescued memory impairment in aged animals even after Aβ plaques were formed.

To investigate the link between slow-oscillations impairment and memory performance, a correlation analysis was performed between the slow-oscillations coherence and the mean latency to the platform in the water maze task for individual animals (figure 12). There was a negative correlation between the latency to the platform and the slow-wave's coherence in single mice, meaning that the impaired slow-oscillations lead to worse cognitive performance in the task.

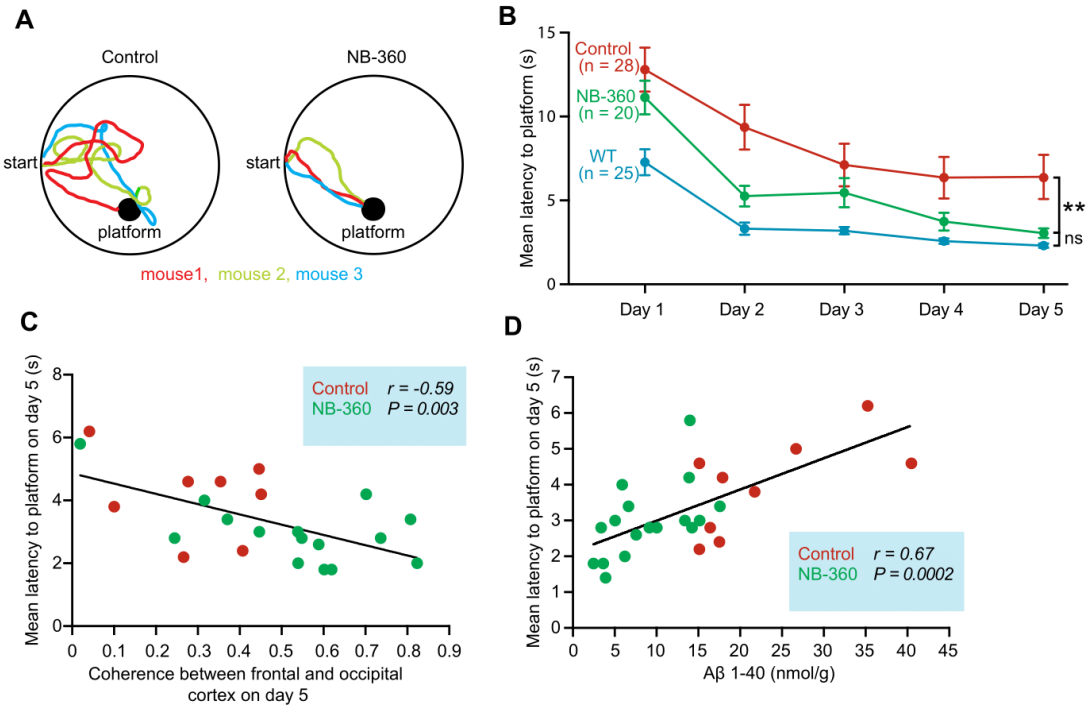

Figure 12: Memory performance is rescued by BACE1 inhibition in transgenic mice. Figure modified from Keskin et al., 2017.

(A) *Schematic showing three exemplary trajectories (each color is one individual) of vehicle-treated APP23xPS45 mice (left) and NB-360-treated APP23xPS45 mice (right) in the water maze task at day five.* ***(B)*** *Summary graph shows mean water maze escape latency of WT (blue, n = 25mice) untreated (red, n = 28 mice), and NB-360-treated (green, n = 20 mice) APP23xPS45 mice (linear mixed effects model, condition: F(2,358)=9.6,P = 8.60*10-5; permutation test, P= <0.001. NB-360vs. WT: t(385)=-1.71, P = 0.0890; permutation test P= 0.108. NB-360 vs. control: t(358)=2.35, P = 0.0193;permutation test P = 0.004). Pearson's correlation coefficient R = 0.65, p = 0.001, Pearson's correlation coefficient R = 0.65, p = 0.001.****(C)*** *The slow-wave activity coherence correlates inversely with memory impairment in APP23xPS45 mice (n=23 mice). Each circle represents an individual animal.* ***(D)*** *FA-extracted Aß levels correlate positively with memory impairment (n = 26 mice). **P < 0.01. 79 = APP23xPS45 model. ns, not significant; p,platform; s, start. Error bars represent mean + SEM.*

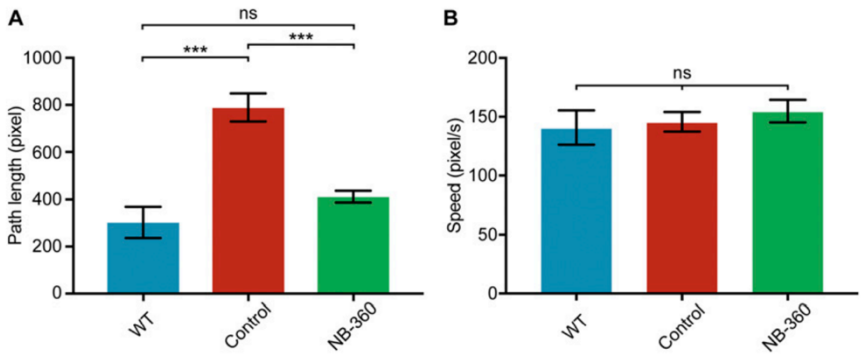

Figure 13: Delayed escape latencies are due to prolonged swimming trajectories and not to motoric deficits. Figure modified from Keskin et al., 2017.

(A) *Summary graph showing path length of WT (blue bar, n=6), untreated (red bar, n=5) and treated APP23xPS45 mice (green, n=5). Path length: 302.3 ± 66.4 pixels for WT vs. 789.2 ± 59.9 pixels for control vs. 412.1 ± 25.4 pixels for NB-360–treated [F (2,13) = 20.68, P < 0.0001; Tukey's post hoc comparisons: P < 0.0001 for WT vs. control, P = 0.0013 for control vs. NB-360, P = 0.3668 for WT vs. NB-360].* ***(B)*** *bar graph shows no difference in swimming speed: 140.8 ± 14.6 pixels for WT vs. 145.7 ± 8.4 pixels for control vs. 154.7 ± 9.6 pixels for NB-360–treated [F(2,13) = 0.3577, P = 0.7059; Tukey's post hoc comparisons: P = 0.9522 for WT vs. control, P = 0.8635 for control vs. NB-360, P = 0.6854 for WT vs. NB-360. ***= P < 0.01]. ns, not significant. Error bars represent mean ± SEM.*

4.6. A potential mechanism of NB-360 – reduction of toxic oligomers

After assessing that the NB-360 rescued different levels of functional impairment in the AD mouse model, it was crucial to identify its mechanism of action. First, after the NB-360 treatment there were substantially fewer Aβ plaques in the brain (figure 5). Although there were remaining Aβ plaques in the brain parenchyma of the treated transgenic mice, the surrounding neurons were normally active. Moreover, lowering of brain Aβ levels correlated with restoring dysfunctions on the cellular (figure 7), long-range (figure 11), and behavioral levels (figure 12).

Anti-Aβ OC antibody staining technique was used to assess the amount of cerebral prefibrillar Aβ (Yuan and Grutzendler 2016). This technique showed that in treated mice, there was a significant reduction of prefibrillar species around existing plaques (figure14). The ratio of OC-stained prefibrillar Aβ to the Thioflavin-S plaque core was significantly diminished in NB-360 treated mice. Thus, instead of Aβ plaques, the prefibrillar Aβ forms were suspected of being toxic and triggering neuronal hyperactivity.

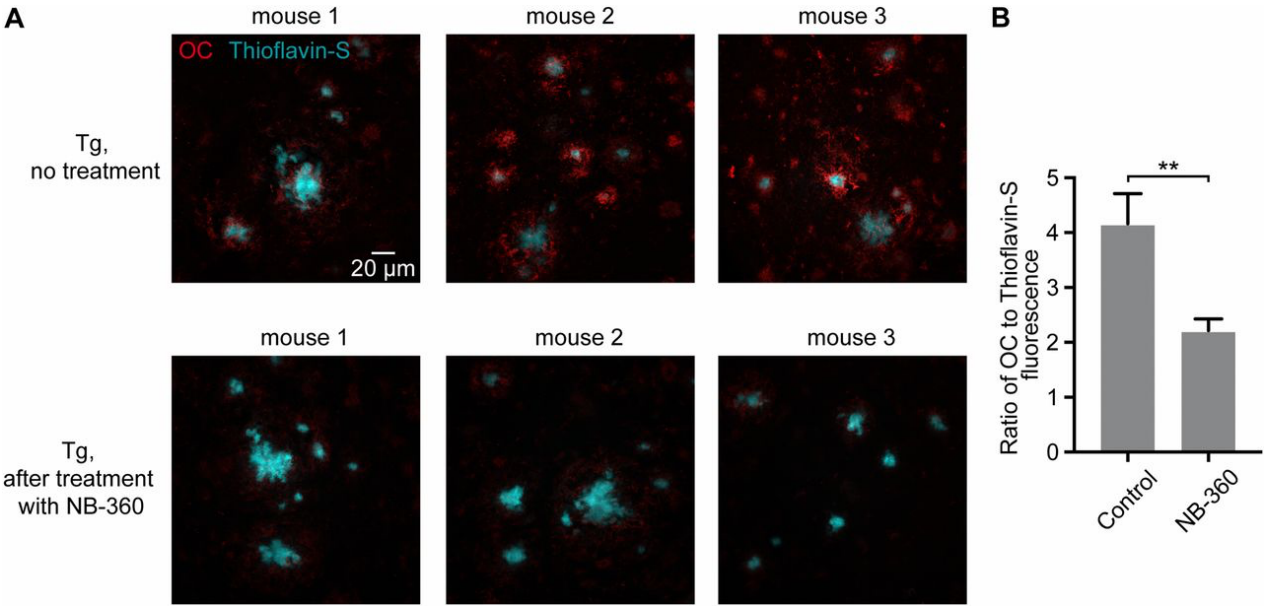

Figure 14: BACE1 inhibition reduces prefibrillar forms of Aβ, which are typically surrounding Aβ plaques. Figure modified from Keskin et al., 2017.

(A) *Confocal images of forebrain sections stained with OC antibody (red) and thioflavin-S (turquoise) from vehicle-treated (top, n= 4 mice) and NB-360-treated (bottom, n=6 mice) APP23xPS45 mice.* ***(B)*** *Bar graph showing a strong reduction of prefibrillar Aδ surrounding Aδ plaques in the NB-360 treated group (4.16 ± 0.55 for control vs. 2.22 ± 0.21 for NB-360; two-sample t test, t = 3.8; df = 8; P = 0.0050). ** = P < 0.01. Error bars represent mean ± SEM.*

Next, I reintroduced soluble Aβ forms after having imaged successfully treated NB-360 mice with no hyperactive cells in the neocortex. The introduction of soluble Aβ forms triggered the resurgence of neuronal hyperactivity (figure 15). Indeed, the topical wash-in of synthetic Aβ dimers in the neocortex of treated transgenic mice revealed local circuit dysfunctions in the form of hyperactivity.

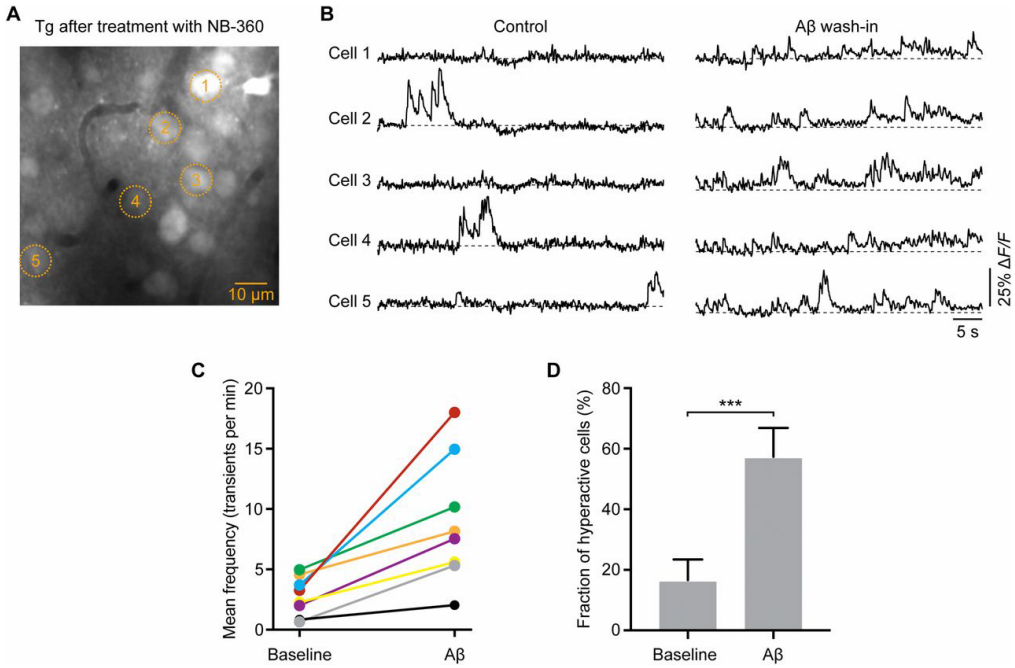

Figure 15: Aβ dimer reintroduction to the cortex of NB-360-treated APP23xPS45 mice provoke the reoccurrence of hyperactivity in neurons. Figure modified from Keskin et al., 2017.

(A) *Image of OGB- AM stained in vivo two-photon image of layer 2/3 neurons in NB-360-treated with 5 marked cells.* ***(B)*** *Ca^{2+} fluorescence traces from left image (A) before (control) and after synthetic AβS26C cross-linked dimers (1 µM) superfusion of the cortex.* ***(C)*** *Mean frequency of Ca^{2+} transients in all imaged regions (n= 8 regions in three mice) during baseline conditions and after Aβ application (2.79 ± 0.57 transients/min baseline vs. 8.98 ± 1.86 transients/min Aβ; paired t test, t = 3.883; df = 7; P = 0.0060).****(D)*** *Fractions of hyperactive cells from all recorded regions (n= 8 regions in three mice) significantly increase after Aβ application compared to baseline conditions (16.62 ± 6.85% for baseline vs. 57.35 ± 9.58% for Aβ; paired t test, t = 5.628; df = 7; P = 0.0008). *** = P < 0.001. Error bars represent mean ± SEM.*

To confirm if soluble Aβ was sufficient to provoke a failure of slow-wave coherence in NB-360 treated mice with restored slow-wave activity, I reintroduced a mixture of

synthetic Aβ1–40 and Aβ1–42 peptides via topical wash-in. Indeed, the peptides triggered a breakdown of brain circuit activity coherence with uncorrelated slow-waves.

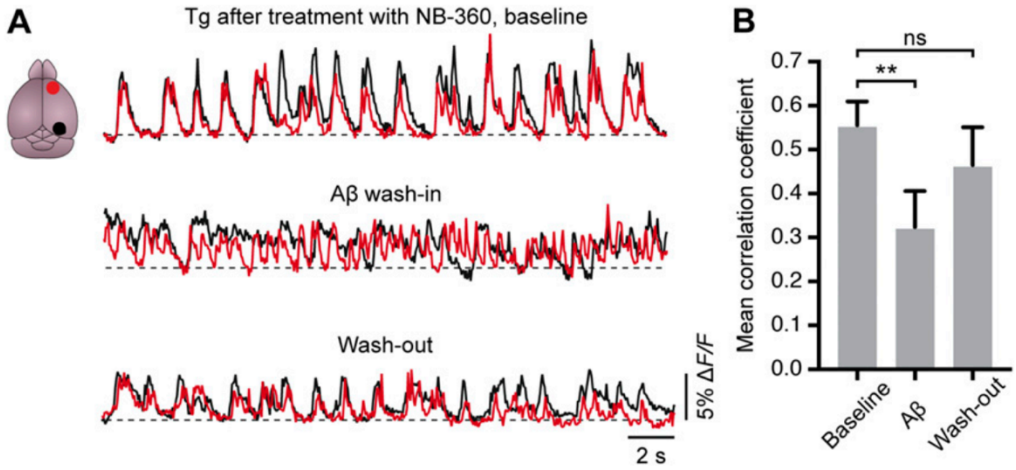

Figure 16: Aβ dimer reintroduction to the cortex of NB-360-treated APP23xPS45 mice provoke the reoccurrence of non-correlated slow-waves.Figure modified from Keskin et al., 2017.

(A) *Exemplary overlayed Ca^{2+} traces of slow-waves in frontal (red) and occipital (black) cortices of a representative, NB-360–treated APP23xPS45 mouse. The top trace shows slow-waves before, the middle trace during, and the bottom trace after introduction with synthetic Aβ 1–40/1–42 (1 μM).* ***(B)*** *The mean correlation strength between the cortices before, during, and after Aβ introduction in NB-360–treated APP23xPS45 (n = 5 mice) mice is represented (0.56 ± 0.05 for baseline vs. 0.32 ± 0.08 for Aβ vs. 0.47 ± 0.09 for washout). There was a significant effect of the treatment [linear mixed-effects model, F(2,11) = 7.07, P = 0.011], and the difference between the baseline condition and the Aβ wash-in was highly significant [F(1,11) = 14.0, P = 0.0032]. **=P < 0.01. ns, not significant. Error bars represent mean ± SEM.*

5. DISCUSSION

The findings of this study support the idea that NB-360 treatment with the BACE inhibitor NB-360 effectively rescues neuronal and circuit dysfunction in addition to spatial memory deficits through the suppression of Aβ production and the subsequent lowering of soluble Aβ species. This study represents a valuable insight demonstrating that neuronal impairments can be restored even in the context of extensive Aβ pathology.

5.1. BACE1 inhibition effectively clears the brain from toxic Aβ species

Several preclinically tested BACE1 inhibitors previously showed excellent efficacy in animal AD models as they effectively lowered Aβ plaque pathology (Kennedy et al. 2016; Neumann et al. 2015; Neumann et al. 2018; Dobrowolska et al. 2014; May et al. 2015; Jeppsson et al. 2012; Sims et al. 2017; Bacioglu et al. 2016; Beckmann et al. 2016; Klohs et al. 2014). In this study, I used the NB-360 compound, which has been shown to keep Aβ at baseline levels in various AD mouse models (Peters et al. 2018; Meier et al. 2018; Bacioglu et al. 2016; Schelle et al. 2017; Neumann et al. 2015). This is consistent with the result of my study in which NB-360 effectively reduces extensive Aβ pathology when mice are already 6-8 months old (mice show extensive amyloid plaque load at this age). Aβ was determined in the brains collected after physiological measurements, during which treated animals had no access to the BACE1 inhibitor. It is known that the treatment's Aβ lowering potential is dose and time-dependent. The NB-360 brain concentration being highest less than one hour after ingestion, Aβ is lowest after four hours (Neumann et al. 2015). The NB-360 mechanism of action explains these Aβ kinetics as the BACE1 inhibitor lowers new Aβ

peptide generation and intrinsic mechanisms clear pre-existing Aβ. Thus measured Aβ levels are likely to be slightly higher than if we had sacrificed the animals directly after taking them out of their home cages, as we conducted imaging experiments under anesthesia before sacrificing them.

5.2. BACE1 inhibition repairs circuit dysfunctions by lowering toxic soluble Aβ species

In this graduate work, I demonstrated that BACE1 inhibition recovers neuronal circuit dysfunctions and behavioral deficits in an AD mouse model by reducing toxic Aβ species.

This study provided experimental evidence that Aβ accumulation accounts for dysfunctions on local and long-range circuit levels, leading ultimately to cognitive impairment. Previously neuronal hyperactivity has been linked to Aβ accumulation (Zott et al. 2019; Zott et al. 2018; Busche and Konnerth 2016; Busche et al. 2012; Busche et al. 2008; Grienberger et al. 2012; Korzhova et al. 2021) but it was not clear if APP overexpression was a confounding variable (Born et al. 2014; Lalonde, Fukuchi, and Strazielle 2012). The current results clarify this link by showing that reducing Aβ in an APP overexpressing model restores physiologic neuronal circuit activity when APP overexpression is still present. The APP knock-in mouse model has been designed to avoid APP overexpression and examine Aβ's effect alone (Saito et al. 2014). Recent studies show circuit dysfunctions in this mouse model and report hyperactivity (Jacob et al. 2019; Johnson et al. 2020; Jun et al. 2020; Arroyo-García et al. 2021; Takamura et al. 2021).

In the past decades, research has tended to focus on Aβ plaques as the main pathology and impairment driver. Here, I demonstrated that soluble prefibrillar Aβ forms

rather than Aβ plaques are responsible for neuronal and circuit dysfunctions. Indeed, in this study, levels of soluble Aβ and the extension of brain circuit dysfunctions (for example abnormal neuronal hyperactivity, slow-wave coherence, spatial memory deficits) are strongly correlated. Chronic inhibition of BACE1 and, therefore Aβ production, restored neuronal circuits activity in the cortex, even if Aβ plaques remained present. Immunohistochemical stainings revealed that the remaining plaques lost their halo of soluble Aβ oligomers. Previous studies confirme the observation that plaques are surrounded by a toxic halo of soluble Aβ oligomers (Koffie et al. 2009; Hefendehl et al. 2016). This provides an explanation, why hyperactive neurons cluster around Aβ plaques in the neocortex of transgenic mice (Busche et al. 2008; Busche, Grienberger, et al. 2015; Grienberger et al. 2012; Liebscher et al. 2016; Rudinskiy et al. 2012; Korzhova et al. 2021). Thus, Aβ plaques are surrounded by a toxic microenvironment attributed to soluble Aβ species,which disrupt synaptic transmission and cellular activity. Moreover, previous data in young transgenic mice showed that hippocampal hyperactivity occurs when soluble Aβ accumulates before Aβ plaque are present, suggesting that plaques are not the primary agent to induce hyperactivity (Busche et al. 2012). Also, acutely inhibiting Aβ peptide production by a γ-secretase inhibitor was shown to restore normal neuronal activity levels in the hippocampus. At the same time, the acute treatment likely did not affect Aβ plaques but instead reduced soluble Aβ species (Busche et al. 2012). Next, in this graduate work, I demonstrated that the reintroduction of Aβ in successfully NB-360 treated mice retriggered neuronal hyperactivity. In addition, previous data showed that local soluble Aβ injections in WT hippocampal cells induced hyperactivity (Zott et al. 2019; Busche et al. 2012). Especially dimers - the smallest Aβ oligomers- are proven to be toxic and to impair synaptic function (Brinkmalm et al. 2019; Yang et al. 2017; Shankar et al. 2008; Li et al.

2009; Walsh et al. 2002; Busche et al. 2012; Zott et al. 2019). For instance, the generation of a mouse model expressing stabilized amyloid dimers and therefore formed no plaques displayed LTP and cognitive deficits just like in the conventional AD mouse models with plaques (Muller-Schiffmann et al. 2016). It is important to note that for the experiment of reinduction of hyperactivity in treated AD mice, I used synthetic AβS26C cross-linked dimers. The crosslink of two monomers is designed to stabilize the dimeric form of Aβ, which is usually unstable and aggregates quickly in a physiologic setting (Shankar et al 2008). However, my colleague confirmed the effect and tested Aβ-containing human brain extract from an AD patient and confrmed the neuronal hyperactivity inducing effects (Zott et al. 2019). For the reinduction of the desynchronization of slow-wave activity, a mixture of synthetically produced Aβ1-40 and Aβ1-42 monomers was used. Monomers are described to have no toxic effect and do not impair synaptic function (Li et al. 2009; Shankar et al. 2008; Walsh et al. 2002; Yang et al. 2017). Nevertheless, monomers tend to oligomerize fast, so we can assume that the Aβ1-40/Aβ1-42 mixture contains active dimers and higher-order oligomers (Nag et al. 2011). Altogether, cumulative evidence indicates that prefibrillar Aβ forms rather than neuritic plaques cause neuronal hyperactivity. This explains clinical findings, where patients bearing Aβ plaques display no cognitive symptoms (Erten-Lyons et al. 2009) and why the amount of amyloid plaque load in patient brains does not correlate well with clinical symptoms (Arriagada et al. 1992).

In this study, NB-360 treatment had a beneficial effect on slow-waves activity and cognition. The impact on cognition is supported by other studies employing a BACE1 inhibitor and seeing cognitive improvement in the AD mice (Chang et al. 2011; Fukumoto et al. 2010). Slow wave coherence was normalized through the NB-360-dependent decrease in toxic soluble Aβ species. As described earlier, slow-wave activity is crucial for

memory formation and cognition and remarkably, restoring slow-wave's activity by either by reducing Aβ as demonstrated here, by GABAergic drugs or optogenetically results in improved memory in AD mouse models (Kastanenka et al. 2017; Busche, Kekus, et al. 2015). Interestingly, restoring slow-waves by transcranial direct current stimulation in early AD patients also improved memory performance (Ladenbauer et al., 2017).

BACE1 inhibition might impact the disease's progression not only by repairing brain circuit activity but also indirectly by normalizing hyperactive circuit activity. First, neuronal hyperactivity has been identified to drive Aβ plaque deposition (Bero et al. 2011; Huijbers et al. 2015; Leal et al. 2017; Yamamoto et al. 2015; Yuan and Grutzendler 2016; Kastanenka et al. 2019). Second, studies provided findings about oscillatory brain circuit activity restoration that prevents Aβ plaque accumulation (Iaccarino et al. 2016; Kastanenka et al. 2017). Therefore, BACE1 inhibition-dependent dampening of excess activity might slow down AD pathology, and this insight raises the possibility of BACE1 inhibitors being a directly disease-modifying drug.

5.3. Neurons are still viable at this advanced stage

In this graduate work, I was able to demonstrate that BACE1 could effectively repair the pathophysiological and cognitive defects described in a mouse model even in the presence of extensive Aβ pathology took place. This was an unexpected finding at that time since other Aβ-lowering therapeutic strategies suggested that chronic pathological processes would irreversibly harm neurons and that those could not recover (Busche, Grienberger, et al. 2015; Das et al. 2001; Mably et al. 2015; Oddo et al. 2004; Yan et al. 2009). Our data imply that most cells in the model are still viable at this advanced pathological stage. Their activity can return to normal levels after the reduction of cerebral

Aβ levels. Previous studies investigating other Aβ targeting treatments such as passive immunotherapies with anti-Aβ antibodies exacerbated neuronal dysfunctions (Busche, Grienberger, et al. 2015). They involved a greater risk for sudden deaths, possibly related to aberrant neuronal activity linked to fatal convulsive seizures (Mably et al. 2015). A possible explanation for the reported discrepancy is the difference in the mechanism of action of the drugs. In the anti-Aβ immunization studies, the tested antibodies failed to clear the brain from the toxic soluble oligomeric forms of Aβ, and one hypothesis was that they would release toxic oligomeric forms from the plaques (Busche, Grienberger, et al. 2015; Mably et al. 2015). BACE1 inhibitors seem to be effective in restoring brain activity as they block the formation of all Aβ forms, including the toxic prefibrillar Aβ surrounding plaques. On a broader level, evidence suggests that therapeutic strategies should focus on clearing AD brains or preventing the formation of prefibrillar Aβ species. In addition, the Aβ plaque loads seem not to be an adequate criterion to measure treatment efficacy. Instead, oligomeric forms of Aβ protein could potentially present a more effective AD biomarker for tracking treatment efficacy (Xia et al. 2009).

5.4. BACE1 inhibitor-dependent side effects

5.4.1. Inhibition of BACE1 impacts substrates other than APP

Since BACE1 is an enzyme that has over 40 substrates other than APP (Yang et al. 2003), a concern is whether BACE1 inhibitors impact the function of other substrates that are involved in essential processes including axon guidance, neurogenesis, muscle spindle

formation, neuronal network functions and myelination (Barao et al. 2016). Although in our study, therapeutically inhibiting BACE1 did not reveal any effect on spontaneous neuronal activity and spatial memory performance in the APP23xPS45 model and spontaneous cortical activity in WT mice, preclinical and clinical trials reported worsened cognition compared to the placebo group (Panza et al. 2019a). This effect caused concerns about a class overlapping effect of adverse events, which could potentially outweigh any positive drug effects on cognition. Preclinical data in line with this observation showed that inhibition of BACE1 activity over 50% affects synaptic health, structure, and functioning in a dose-dependent manner (Satir et al. 2020; Filser et al. 2015; Zhu, Xiang, et al. 2018; Lombardo et al. 2019). Indeed, several BACE inhibitors (SCH1682496, LY2811376, and C3) were shown to impair synaptic transmission similarly to the BACE kock-out phenotype (Kamikubo et al. 2017; Filser et al. 2015). Interestingly, the knock-out of a single BACE1 allele resulted in no impairment, indicating that an BACE1 inhibition of over 50% is deleterious (Giusti-Rodriguez, 2011 #566).

One candidate BACE1-substrate, which might be responsible for the described side-effects is SEZ6. Its lack of shedding and knock-out compromises spine formation, density, hippocampal LTP, and cognition in the WT mice (Filser et al. 2015; Zhu, Xiang, et al. 2018; Gunnersen et al. 2007). The observed reduced spine density in NB-360 BACE1 inhibitor-treated WT mice results from impaired new spine formation (Filser et al. 2015; Blume et al. 2018; Zhu, Peters, et al. 2018). The structural and functional synaptic plasticity, which is at the cellular basis of cognition is therefore impaired during BACE1 inhibition and might be a potential explanation for the observed cognitive deficits in human trials. Further preclinical studies demonstrated that the effect of BACE1 inhibition on the plasticity of dendritic spines strongly depends on the dosage of the BACE1 inhibitor. Whereas high inhibitor

concentrations caused a decrease in spine plasticity, this effect was not observed at lower drug concentrations, which decreased the Aβ-level only by 40% (Filser et al. 2015).

NRG1 is another BACE1 substrate that binds to the erbB4 receptor and has a similar effect as SEZ6. The NRG1-erbB4 signaling regulates synaptic plasticity and cortical interneuron formation via postsynaptic glutamate receptors (Li et al. 2007). Genetic deletion or severe inhibition of BACE1 decreases NRG1 cleavage and thus impairs NRG1-erbB4 signaling, destabilizing postsynaptic glutamate receptors. These impairments reduce synaptic plasticity and ultimately lead to a loss of dendritic spines (Huang et al. 2000; Li et al. 2007).

Another relevant BACE1 substrate candidate is CHL1, crucial for axon guidance in the hippocampus, a structure essential for the cognition (Ou-Yang et al. 2018). A conditional knock-out in adult mice results in severe axon guidance and organization disruption in the hippocampus's mossy fiber pathway, suggesting long-term BACE1 inhibition could also disturb axonal structures (Ou-Yang et al. 2018). CHL1 seems to play a role in synaptic functioning as its loss impairs LTP (Nikonenko et al. 2006).

5.4.2. BACE1 INHIBITION AFFECTS PROTEOLYTIC APP FRAGMENTS

BACE1 inhibition blocks the amyloidogenic pathway and shifts proteolytic activity towards the non-amyloidogenic and alternative APP processing pathways, increasing the secretion of fragments such as sAPP-α and Aη-α (Dobrowolska et al. 2014; Neumann et al. 2015; Fukumoto et al. 2010). This sheds light on a possible unwanted side-effect of therapeutical β-secretase inhibitors.

Evidence suggests that secreted proteolytic APP fragments are responsible for the synaptic function of APP (Mockett et al. 2017; Muller, Deller, and Korte 2017). They represent an essential portion of almost 50% of total APP forms in the brain (Morales-Corraliza et al. 2009). The released sAPP-α fragment is well-known to be neuroprotective as it activates survival pathways, upregulates protective molecules, inhibits caspase-dependent apoptosis, and reinforces neuronal resistance to injuries (Hefter et al. 2016; Kogel, Deller, and Behl 2012). Interestingly, it has other beneficial properties: a study showed that by binding to BACE1, sAPP-α blocs BACE1's activity and thus tips the balance towards the non-amyloidogenic pathway, preventing toxic Aβ formation (Obregon et al. 2012). In addition to the neuroprotective characteristics, sAPP-α plays a decisive role in synaptic plasticity, learning, and memory. Indeed, exogenously applying sAPP-α was shown to facilitate LTP (Taylor et al. 2008; Ishida et al. 1997). Moreover, sAPP-α possibly facilitates LTP via de novo synthesis of glutamate receptors and their trafficking to the cell surface (Mockett et al. 2019). The positive effect of APPα was demonstrated *in vivo* as sAPP-α infusions in aged mice rescued cognitive deficits (Xiong et al. 2017). It appears that sAPP-α is involved in the regulation process of protein synthesis in dendrites (Claasen et al. 2009) and inversely, exogenous sAPP-α application or sAPP-α knock-in APP knock-out mice restored reduced spine density (Weyer et al. 2011; Weyer et al. 2014). Viral AAV-mediated expression of sAPP-α in the brains of APP/PS1 rescued/improved synaptic function and structure as well as cognitive deficits and reduced soluble Aβ levels and plaque load (Fol et al. 2016). Therefore a BACE1 inhibitor-dependent increase of sAPP-α might be beneficial.

BACE1 inhibition reduced the secretion of fragments such as sAPP-β and p3. sAPP-β does not modulate LTP or display neuroprotective effects, suggesting that its treatment-related reduction would not affect the synaptic functioning (Mockett et al. 2019; Hick et al.

2015; Taylor et al. 2008). Through BACE1 inhibition and the enhancement of non-amyloidogenic processing, the p3 peptide secretion increases. This peptide is not well studied but considered mainly benign (Kuhn and Raskatov 2020). P3 was shown not to affect synaptic function (Walsh et al. 2002). However, future studies need to elucidate its function, since p3 represent a major component of diffuse plaques (Gowing et al. 1994; Saido et al. 1996; Lalowski et al. 1996; Higgins et al. 1996) and is detected in dystrophic neurites adjacent to plaques in AD brains (Higgins et al. 1996).

The recently discovered alternative APP processing pathway is particularly interesting because it secretes the synaptotoxic peptide Aη-α. Indeed, BACE1 inhibition results in the accumulation of Aη-α, which suppresses synaptic plasticity *in vitro* and neuronal activity *in vivo*, resulting in neuronal silencing (Willem et al. 2015). As mentioned before, I did not observe any effect on activity levels in NB-360 treated WT animals, such as an increase in neuronal silencing. The used drug dosage was lower in my study than in the paper describing the effects of Aη-α. Moreover, they used a single dose of BACE1 inhibitor that induced LTP impairments. Therefore it could be that in our chronic treatment, adaptive mechanisms took place.

As discussed, I did not see any impairment of neuronal activity after BACE1 inhibition in this graduate work. However, the phenotype might have been too subtle to be detected with Ca^{2+}-imaging and would require more detailed electrophysiological experiments. Another possibility might be that the treatment timeframe was not long enough to observe the phenotypic outputs, which is not likely, since the side effects in other studies were reported after 2-3 weeks of treatment (Blume et al. 2018; Filser et al. 2015). Moreover, different BACE1 inhibitors have been used in preclinical studies, but studies using our NB-360 also reported those synaptic disruptions (Peters et al. 2018; Zhu,

Peters, et al. 2018; Zhu, Xiang, et al. 2018). As mentioned before, the side effects were not observed at lower drug concentrations (Filser et al. 2015). The further detailed analysis could include the CSF proteome measuring BACE's substrates, APP's proteolytic fragments, and potential new substrates. This would deepen our understanding of the mechanism behind the reported BACE-dependent side effects, and the CSF proteome may serve as biomarkers to monitor BACE1 inhibitor treatments. Moreover, electrophysiological experiments could elucidate in the *in vivo* setting the neurophysiological mechanism behind the BACE-1 inhibition (Dislich et al. 2015). It is especially crucial to investigate the treatment's effect in the acute and chronic phase and perform a dose-dependent analysis, which would reveal treatment dosages, where the described side effects are manageable.

Concerning other side effects unrelated to cognitive functions, I could observe fur discoloration in treated animals. This can be explained as NB-360 is not specific for BACE1 but also blocks BACE2, which is its close homolog (Sun et al. 2005; Bennett et al. 2000; Neumann et al. 2015). BACE2 processes the pigment cell-specific melanocyte protein involved in melanosome formation in pigment cells, and therefore, its inhibition results in fur discoloration (Shimshek et al. 2016; Rochin et al. 2013). At this point, it is important to mention that BACE2 is mainly expressed in peripheral tissues and is almost absent in the human fetal or adult brain (Voytyuk et al. 2018; Bennett et al. 2000). Overall, the BACE2 function seems to be different from BACE1, and their functions are different for tissues and cell types. However, there is a possibility for BACE2 to overtake to some extend APP's processing, even though its share can be neglected (Vassar et al. 2014; Voytyuk et al. 2018).

Overall, despite the beneficial blockage of the amyloidogenic pathway, BACE1 inhibitors have been shown to compromise synaptic function in the experimental setting via the induction of alternative APP pathways and the inhibited processing of other

physiologic BACE1 substrates. These findings might explain the cognitive worsening seen in clinical BACE1 inhibition trials at least to some extend (Panza et al. 2019b). Altogether, these results raise the need for monitoring levels of alternative BACE1 substrates, proteolytic APP fragments in relation to CSF Aβ levels to assess the potential toxicity and the efficacy of the treatment.

5.5. Potential reasons for failed clinical BACE1 inhibitor trials

BACE1 inhibitors were a source of great hope for researchers, doctors, AD patients, and their families. Despite the strong preclinical support, none of the five major Phase III-tested BACE1 inhibitors was able to show clinical benefit in MCI (mild cognitive impairment – the stage before AD diagnosis, where cognitive symptoms start to appear), mild, and mild-to moderate AD patients or individuals at risk of developing AD (Das and Yan 2019). Large phase III clinical studies failed to show cognitive improvement or a slowing of cognitive decline despite strong target engagement and effective Aβ reduction (table 2). Moreover, study results revealed side-effects such as a transient cognitive worsening or brain atrophy leading to premature interruption of those trials (Panza et al. 2019b). Two main factors possibly account for the reported clinical trial failures: the treatment time point and mechanisms related to side-effects.

Table 2: BACE1 inhibitor clinical trials.

Agent (Sponsor)	Trial (Phase)	Patients	Main outcomes	References
Verubecestat (Merck)	EPOCH (III)	Mild-to-moderate AD	No cognitive improvement Brain volume loss	(Egan et al. 2018; Egan et al. 2019; Sur et al. 2020; Cummings 2018; Wessels, Tariot, et al. 2020)
	APECS (III)	Prodromal AD	Cognitive worsening	
Lanabecestat (Eli Lilly /AstraZeneca)	AMARANTH (III)	MCI to mild AD	No cognitive improvement Improvement in subdomains Brain volume loss	(Wessels, Lines, et al. 2020; Zimmer et al. 2021))
	DAYBREAK-ALZ (III)	Mild AD		
Atabecestat (Janssen)	EARLY (II/III)	Asymptomatic at risk	Cognitive worsening Liver toxicity	(Henley et al. 2019; Sperling et al. 2021)
Umibecestat (Amgen/Novartis)	GENERATION1/2 (II/III)	Asymptomatic at risk	Cognitive worsening Brain volume loss	(Graf et al. 2020; Reiman et al. 2020)
Elenbecestat (Esai/Biogen)	MISSION AD 1/2 (III)	MCI	No cognitive improvement	(Rogers 2021)

5.5.1. A WINDOW OF OPPORTUNITY FOR BACE1 INHIBITION

BACE1 inhibitors operate via blocking the Aβ production and enabling physiologic clearing processes to reduce existing accumulations (Vassar 2014). As introduced before, cerebral Aβ deposition is an early process that appears to begin decades before the pathologies' clinical manifestation in the form of cognitive deficits (Musiek and Holtzman 2012). Thus Aβ lowering therapeutic strategies are most likely to be effective as a prevention strategy before significant cerebral amyloid accumulation and neurodegeneration takes place. This is also true for secondary prevention when Aβ is below

a critical concentration, which would induce the pathological cascade leading to neurodegeneration and cell loss. Thus, it is strategically important to define a time window for when the Aβ-lowering treatment is still effective. First, it has been shown that BACE inhibitors are more effective at preventing the formation of Aβ plaques than their removal (Das et al. 2012; Hu et al. 2018). Therefore, Aβ-lowering treatments are more likely to be effective at early stages, when the pathology is limited. Second, the window of opportunity is described as the stage at which cerebral damage is limited and cognitive impairment can be prevented or reversed**.** The AD pathogenesis is thought to progress through an amyloidogenic phase where amyloid mainly accumulates, and later on, tau joins and overtakes. The most significant structural correlate of cognitive decline is synaptic loss and neurofibrillary tangles rather than Aβ plaques (Arriagada et al. 1992; Wilcock and Esiri 1982; Bennett et al. 2004; Terry et al. 1991). It can thus be suggested that tau is a likely candidate inducing synaptic loss. Tau is shown to combine its toxic properties with Aβ so that their combination is lethal for surrounding synapses (Ittner and Gotz 2011), standing for the trigger and the bullet in the AD pathogenesis (Bloom 2014). However, the exact mechanisms by which tau promotes toxicity is not clear yet. A recent study in transgenic mice bearing both Aβ and tau accumulations revealed that, once both pathologies were combined, neuronal dysfunctions could not be recovered by lowering tau even in young mice before neurodegeneration occurred (Busche et al. 2019). This result provides the basis for the hypothesis that once soluble tau accumulates on top of Aβ, cerebral function, which is the basis for cognition, is severely damaged and cannot be rescued. This factor may explain the lack of clinical improvement after Aβ suppression in recent Aβ lowering clinical trials in subjects with AD that may already bear (soluble) tau species. BACE1 inhibitor trials have been conducted in individuals with mild cognitive impairment, prodromal, and mild

to moderate AD. At these stages, tau already propagates into cortical areas where Aβ plaques accumulate and marks the transition from preclinical to clinical AD (Wang et al. 2016; Pontecorvo et al. 2017; Johnson et al. 2016; Mufson, Ward, and Binder 2014). The MCI AD stage would therefore be too late for effective BACE1 inhibitor therapy. According to this theory, treatment should begin even earlier, when only Aβ accumulates, and hyperactivity-linked memory impairment can be reversed.

5.5.2. The study models the early preclinical phase of AD

The mouse model I used in my graduate work modeled the amyloidogenic phase of AD, and I studied its functional impact on brain activity. The mouse model simulates the preclinical AD stage with high cerebral Aβ load, but without tau pathology, neurodegeneration (De Strooper and Karran 2016; Sasaguri et al. 2017). The APP23xPS45 translational mouse model bridges basic research with clinical application. Thus the hyperactivation of brain areas in early AD is linked to Aβ deposition and is an early biomarker to identify individuals in the preclinical phase of AD (Corriveau-Lecavalier et al. 2019). As the disease progresses, brain activity decreases, and late-stage AD patients display less brain activity (Dickerson et al. 2005; Huijbers et al. 2015; O'Brien et al. 2010). Although the mouse model used in this thesis seems to reflect findings from the early AD preclinical phase where Aβ dominates, it is important to recognize that the AD pathology even at the early stage involves several other possibly accumulating proteins including APP, secreted APP fragments, Aη, and later Tau that complicates the picture (Harris et al. 2020). Therefore, it is challenging to directly translate these preclinical findings to clinical AD, especially as the disease progresses to different phases that are different from one to another. However, our results suggest that BACE1 inhibitors represent a promising

treatment strategy in early preclinical AD when Aβ dominates and before massive neurodegeneration occurs.

The clinical trials EARLY, testing atabecestat followed this prevention paradigm and recruited healthy individuals at risk for AD, meaning that they were asymptomatic amyloid-positive subjects. These were selected with a CDR staging of 0 and confirmed amyloid positivity by PET or CSF. Still, the study was stopped prematurely because of liver toxicity and an unbeneficial risk-benefit ratio (Henley et al. 2019). When analyzing, the authors did not see any improvement in cognition and even reported cognitive worsening in the highest dose group (Sperling et al. 2021). In order to reconcile the findings of my preclinical study with the lack of effect in the EARLY trial, one has to notice that the atabecestat treatment might not have been long enough. Indeed, the trial has been terminated at 18 months, which might be too early to see its preventive effect on cognitive decline, which is rather a late-stage AD symptom. Therefore another BACE inhibitor with fewer side effects or another dosage might be needed.

Taken together, my preclinical results and the discussed literature support the idea that Aβ lowering therapies may be effective in the specific time window when only Aβ is accumulating, and no other downstream biomarkers and neurodegeneration happen.

5.5.3. An early low-dose treatment

A main roadblock for BACE1 inhibitors was the reported side effects in clinical trials. Cognitive worsening was reported in three out of five tested drugs (verubecestat (Sur et al. 2020), umibecestat (Reiman et al. 2020), atabecestat (Sperling et al. 2021)). A possible reason for the observed worsening of cognition could be the mechanistic BACE1 inhibitor side effects. Indeed, preclinical models show that BACE1 inhibition over 50% negatively

affects synaptic health, structure, and functioning in a dose-dependent manner (see chapter 1.5) (Satir et al. 2020; Filser et al. 2015; Zhu, Xiang, et al. 2018; Lombardo et al. 2019). In order to minimize BACE1 inhibition-related side-effects, the use of a dose range that balances acceptable mechanism-related side effects with an efficient Aβ reduction is needed. The strong Aβ lowering effect seen in clinical trials seems to result from strong BACE1 inhibition (>70% BACE1 inhibition), and therefore, mechanistic side effects are conceivable (Hampel et al. 2021; McDade et al. 2021). Such substantial Aβ reduction and BACE1 inhibition might not be necessary for AD's preclinical or prodromal stage, where the amyloid pathology is still limited. Also, it has been described that the protective Icelandic mutation reduces the BACE1 activity by approximately 50%, resulting in a ~30% reduction of APP's BACE1 cleavage and Aβ production (Jonsson et al. 2012; Martiskainen et al. 2017). This observation would seem to suggest that reducing Aβ production only partially would prevent or delay AD development if started early enough (Jonsson et al. 2012; Benilova et al. 2014; Maloney et al. 2014; Cheret et al. 2013). Experimental results confirmed those observations, as knocking out one allele of BACE1 in AD mice models a 50% therapeutic inhibition and showed ~20% Aβ lowering (Laird et al. 2005; McConlogue et al. 2007). This change was sufficient to induce a dramatic reduction in Aβ plaques, neuritic burden, synaptic deficits, and cognitive deficits in older mice. When it comes to more advanced AD cases, higher doses of BACE1 inhibition might be necessary if significant amyloid plaque load is present. At this point, it is important to mention that BACE1 levels and activity are increased in AD patients (Yang et al. 2003; Li et al. 2004) and that both BACE1 and APP cluster in dystrophic neurites around amyloid plaques (Sadleir et al. 2016; Kandalepas et al. 2013). Therefore, higher doses of BACE1 inhibitors might be needed to block elevated BACE1 concentrations in individuals with a high amyloid burden, increasing the risk for

unwanted BACE1 related side effects (Peters et al. 2018). Indeed, experimental data indicates that lower doses are needed to block the formation of plaques than to clean the brain from plaques. This would imply, that lower doses could be used in presymptomatic patients who do not have a high plaque load yet. A pessimistic scenario would be that once a certain threshold of Aβ is reached, Aβ would kick off a cascade of pathologies that subsequent BACE1 inhibition could not stop or delay. Therefore the earlier the BACE1 inhibition therapy would start, the less drug dosage and side effect would be needed and the more efficient it would be. However, if BACE1 inhibitors would be used as long-term preventive medication its other side-effects such as transient cerebral atrophy, hepatotoxicity, anxiety, weight loss, falls and injuries, suicidal ideation, and sleep disturbances have to be equally investigated and minimized in order to balance the risks and benefits appropriately (McDade et al. 2021).

To better translate our findings into clinical research, an improved stratification of patient populations according to their AD phase and optimal dosage of BACE inhibitors, balancing the benefit versus side effects, would be needed. To obtain an accurate patient stratification, including objective functional biomarkers such as fMRI, BACE1 enzyme activity analysis in the diagnostical routine would be beneficial. Moreover, an improved objective diagnostical process would raise the possibility of individually tailored treatments of patients according to their disease stage in the future. Lastly, the possibility of a preventive population-wide treatment in the elderly seems to be premature at the moment, as more research into the adverse effects of BACE inhibitors and their reversibility is necessary.

6. PUBLICATION

Keskin, A.D., Kekus, M., Adelsberger, H., Neumann, U., Shimshek, D.R., Song, B., Zott, B., Peng, T., Forstl, H., Staufenbiel, M., Nelken, I., Sakmann, B., Konnerth, A., Busche, MA. 2017. BACE inhibition-dependent repair of Alzheimer's pathophysiology. Proc Natl Acad Sci U S A 114: 8631- 36

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