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The effect of BACE1 inhibition in the amyloid plaque-associated axonal dystrophies

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Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Jochen Herms betreut und von Herrn PD Dr. Stylianos Michalakis von der Fakultät für Chemie und Pharmazie vertreten.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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2. Gutacher: Prof. Dr. Jochen Herms

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Dedicated to my grandmother,

Avó Maria

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List of abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
AIDC	APP intracellular domain
APP	Amyloid Precursor Protein
ApoE	Apolipoprotein E
APLP	APP-like protein
BACE1	β-site APP enzyme 1
BACE2	β-site APP enzyme 2
BBB	Blood-brain barrier
βCTF	C-terminal fragment, named C99
CNS	Central nervous system
CSF	Cerebrospinal fluid
eGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic reticulum
FAD	Familial AD
LAMP1	Lysosomal-associated membrane protein 1
NFTs	Neurofibrillary tangles
PB	Sodium phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSEN	Presenilin
SORL1	Sortilin-related receptor 1
TGN	Trans-Golgi-network
TREM2	Triggering receptor expressed on myeloid cells 2
3D	Three dimension

Summary

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease pathologically characterized by the presence of neurofibrillary tangles (NFTs), neuropil threads and dystrophic axons. Dystrophic axons are associated with extracellular depositions of amyloid β (Aβ), known as plaques and present as swollen tortuous neurites, with variable morphology and composition depending on the pathological stage of AD. Among other mechanisms, the presence of dystrophic axons has been observed to contribute to the synaptic alterations in AD.

Moreover, axonal dystrophies are enriched with amyloid precursor protein (APP) and β-site APP enzyme 1 (BACE1). The accumulation of these two proteins results from localized defects in axonal lysosome transport and maturation leading to an increase of BACE1 levels and processing of APP. Thus, over the time, it leads to an Aβ overproduction, exacerbating the formation of dystrophic axons and the progression of amyloid pathology in AD.

For these reasons, BACE1, as the enzyme that initiates the amyloidogenic pathway, is a promising therapeutic target for reducing Aβ levels. We performed chronic two-photon *in vivo* imaging and immunohistochemistry to analyze the effect of pharmacological BACE1 inhibition on the amyloid pathology in the cerebral cortex of AD transgenic mice. We observed that after BACE1 inhibitor treatment, the plaque growth rate decreased notably and majority of the analyzed dystrophic axons were recovered. Furthermore, during the 3 months of BACE1 inhibitor treatment, the formation of new dystrophies was not observed.

Based on our results, BACE1 inhibition might be a therapeutic opportunity for limiting the amyloidogenic processing of APP at dystrophic axons and the development of axonal pathology in close proximity to amyloid plaques contributing to the disease progression.

INTRODUCTION

1. Alzheimer's disease

1.1. Definition and history

AD is the most common age-related neurodegenerative disease. While there is no cure for the disease, it is one of the most devastating diagnoses given to the patients and their families.

Clinically, AD is characterized by memory deficits and progressive loss of cognitive abilities. Memory decline initially manifests as a loss of episodic memory, which is considered to be a selective defect of declarative memory. At first, the dysfunction in episodic memory impedes the recollection of recent events including autobiographical activities. Gradually, memory is lost along with cognitive abilities such as orientation, language, problem solving, calculation and visuospatial perception. In late stages, AD patients are mentally and physically dependent on others for care (Jucker et al. 2006).

Alois Alzheimer (Figure 1) identified the first case of AD in the early part of the 20th century. In 1901, Auguste Deter (Figure 1), a 51-year old woman who had developed memory deficits, progressive loss of cognitive abilities and psychiatric symptoms was examined by Alzheimer. Due to her middle age and the progression of her behavioral symptoms, Alzheimer conducted several investigations to study Deter's case. Five years later, when she died, Alzheimer performed an autopsy that revealed the histological alterations in the cerebral cortex. The analysis of her behavioral symptoms together with the isolation of pathological alterations, gave the name to this condition known as Alzheimer's disease (M. B. Graeber and Mehraein 1999; Manuel B. Graeber 1999; García-Marín, García-López, and Freire 2007; Jucker et al. 2006).

Figure 1: Identification of Alzheimer's disease. Alois Alzheimer (left) and his first diagnosed patient with AD, Augusta Deter (right). Public domain photos.

1.2. Pathological hallmarks

Pathologically, AD is characterized by three abnormalities of the brain, which were described by Alzheimer macroscopically and microscopically.

Macroscopically, the brain is atrophied, with widened sulci, reduced weight and enlarged ventricles. These alterations do not occur uniformly throughout the brain, but affect notably specific regions. The hippocampi, the neocortex, particularly the temporal and frontal lobes, are especially vulnerable.

Microscopically, the major neuropathological hallmark of AD is the extracellular deposition of amyloid plaques formed by aggregates of Aβ. These extracellular deposits of Aβ are surrounded by swollen axons and dendrites (Figure 2). Moreover, the remaining affected neurons have cytoskeletal abnormalities, which consist of intracellular accumulation of NFTs (Figure 3). These tangles contain paired helical filaments formed by hyperphosphorylated tau (a microtubule-associated protein).

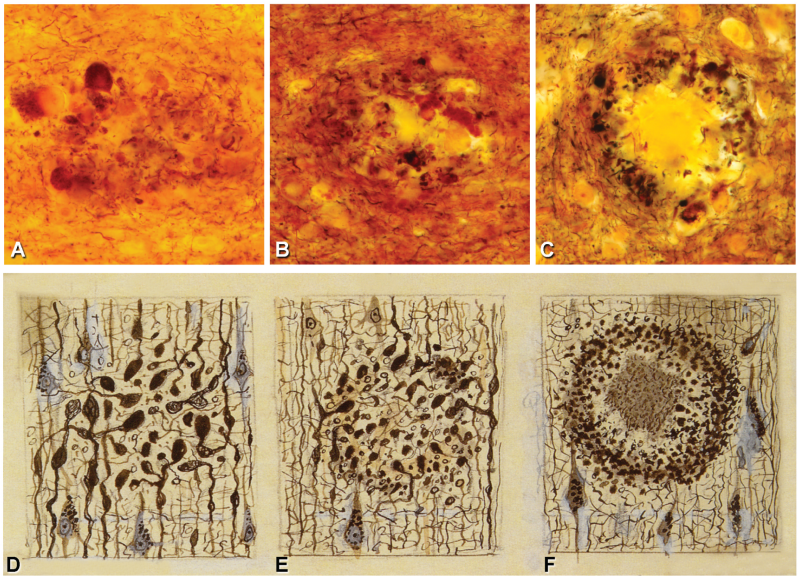


Figure 2: Stages of classical senile plaque formation in the cortex. (A-C) Cajal's histological preparations with the reduced silver nitrate methods and (D-F) original drawings to represent his discoveries. Figures adapted from (García-Marín, García-López, and Freire 2007).

As AD progresses, the reduction in brain volume, especially in the entorhinal cortex and hippocampus, results from the prominent synapse loss and neuronal cell death. These alterations in the entorhinal cortex and hippocampus are the base of the selective defect in declarative memory, which is the first symptom of AD (Manuel B. Graeber 1999; M. B. Graeber and Mehraein 1999; Jucker et al. 2006).

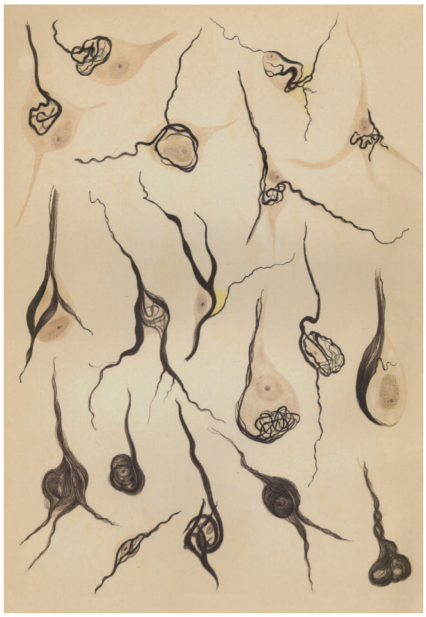


Figure 3: Drawings of neurofibrillary tangles stained with silver impregnation method performed by Sala (1913). Figure adapted from (Javier DeFelipe 2009).

1.3. Epidemiology

AD is the dominant cause of dementia worldwide during aging processes. By the age of 85, between 25-40% of the people have developed AD, and on a global scale there are an estimated 46 million people living with dementia (Alzheimer's Association 2010; Alzheimer's Association 2012; Alzheimer's Association 2015; Wimo, Winblad, and Jönsson 2010; "World Alzheimer Report 2015: The Global Impact of Dementia | Alzheimer's Disease International" 2016). Therefore, as the human population ages it is predicted that over 100 million individuals will have AD in 2050.

Currently, there is no treatment for curing AD, only treatments to relief both cognitive and behavioral symptoms temporally. As the number of AD patients is growing, researchers are investing to find new effective treatments to slow down, prevent or even stop the progression of the AD. This turns in a global economic impact in order to improve the quality of life for patients and their families.

AD patients have a life expectation between 7-10 years on average, although some of them can live for 20 years from the time of diagnosis to death. Moreover, clinical symptoms are gradual and progressive and can differ between patients. Considering this, AD typically progresses in three stages (Figure 4): mild (early-stage), moderate (middle-stage) and severe (late-stage) (Feldman and Woodward 2005). In an early stage of AD, the disease starts progressing with a low accumulation of Aβ without causing symptoms. This is called the preclinical stage of the disease. As Aβ accumulation increases abnormally and the NFTs accumulate in neurons, they lose their ability to function efficiently. This begins in the hippocampus and entorhinal cortex, the brain areas responsible for forming memories. The early symptomatic stage of AD is called mild cognitive impairment. The peculiar functional problems of AD patients are the signs and symptoms of moderate AD, which result from the progressive neuronal dysfunction and dead. As more neurons die, the affected brain regions begin to atrophy. By the late stage of the disease, neuronal damage is widespread leading to a significant shrinkage of the brain. AD patients are unable to communicate and are completely dependent on the others for their care.

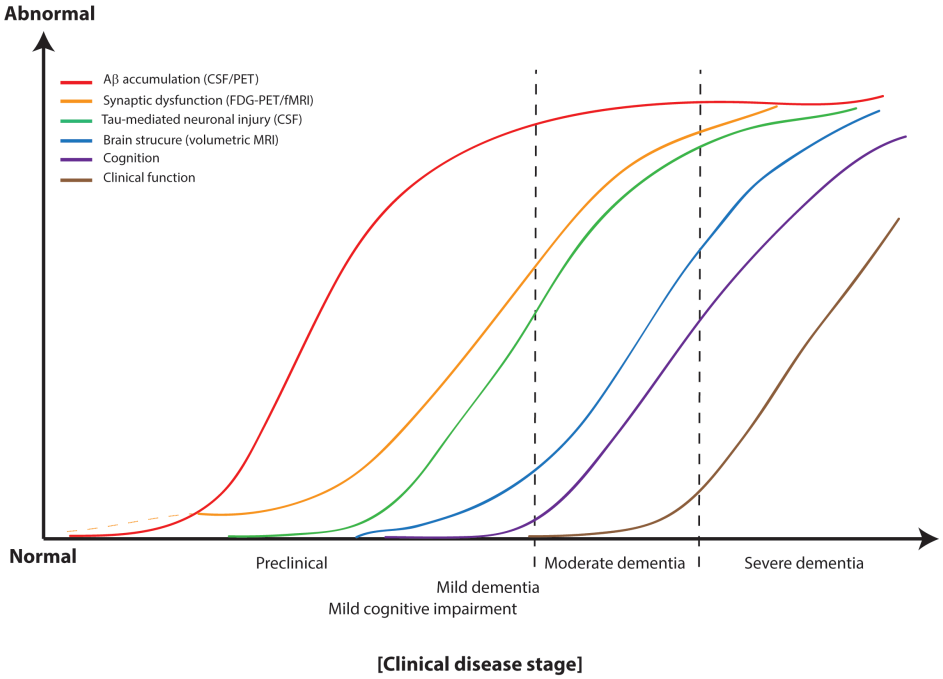


Figure 4: Hypothetical model of dynamic biomarkers of the AD expanded to elucidate the clinical disease stage. Biomarkers change from normal to abnormal (y-axis) as a function of disease stage (x-axis). Figure adapted from (Sperling et al. 2011).

1.4. Genetics of Alzheimer's disease

Memory loss, difficulty performing daily activities, behavior changes, etc., are common symptoms of AD patients. Although the symptoms and pathological characteristics are similar (Geert Van Gassen and Annaert 2003), there are two main categories of AD: familial and sporadic. Familial AD (FAD) represents less than 1% of all AD cases and is associated with heritable autosomal dominant mutations (G. Van Gassen and Van Broeckhoven 2000; Morris et al. 2012). The majority of AD cases are sporadic, without a particular genetic cause, beginning after age 65 and older.

Mutations in three genes have been implicated in FAD: APP, Presenilin (PSEN) 1 and PSEN2 (Goate et al. 1991; Levy-Lahad, Wasco, et al. 1995; Levy-Lahad, Wijsman, et al. 1995; Rogaev et al. 1995; Sherrington et al. 1995; Sherrington et al. 1996). FAD patients have an earlier onset and faster progression of the disease compared to sporadic AD with the first symptoms manifesting approximately by the age of 40 (Rosenberg 2000). In general, these mutations lead to an increase of Aβ production, particularly Aβ42 (Johnston et al. 1994; Suzuki et al. 1994).

All FAD APP mutations influence the proteolytic processing of APP leading to an increase of Aβ generation, aggregation and plaque formation as they are positioned in or near the Aβ-coding exons (16 and 17) of APP which encode the Aβ sequence (Haass et al. 1994; Ishii et al. 2001; Schellenberg and Montine 2012).

Most of PSEN mutations are single-nucleotide substitutions in subunits of the γ-secretase enzymatic complex, which cleaves APP into two amyloid peptides (Aβ42 and Aβ40) of different lengths. Biochemically, these mutations result in a partial loss of function in the γ-secretase complex (B. De Strooper et al. 1998) but from a genetic point of view, they result in a gain of toxic function. As a consequence, mutations in either PSEN or APP result in an increase of the ratio between Aβ42/Aβ40 by an increase in Aβ42 and/or a decrease in Aβ40 (Scheuner et al. 1996; Borchelt et al. 1996).

In the vast majority of AD cases, genetic risk factors increase the susceptibility for developing AD, though they are not the main cause. Neither genetic nor environmental factors acting separately can cause sporadic AD, both are necessary but not sufficient for the late-onset disease development.

A large number of common variants or polymorphisms in different genes of AD patients have been identified as risk factors that can contribute to the development of sporadic AD. Genomic studies found several genes involved but among them the most relevant risk factor is the polymorphic gene encoding apolipoprotein E (ApoE) (Strittmatter, Saunders, et al. 1993; Rocchi et al. 2003). When the risk-conferring allele (ApoEε4) is present in heterozygous individuals it increases three-fold the risk for developing AD, while homozygous increase AD risk by 12-fold (Corder et al. 1993; Strittmatter, Saunders, et al. 1993; Strittmatter, Weisgraber, et al. 1993).

ApoE binds to Aβ influencing its degradation and clearance. Aβ degradation is influenced by ApoE isoform status through indirect mechanisms such as interaction with receptors/transporters responsible for endocytosis and lysosomal degradation. Thereby, ApoE sequesters Aβ preventing its clearance which results in a accelerated Aβ deposition and increases the rate of AD pathology (J. Kim, Basak, and Holtzman 2009; Castellano et al. 2011; Verghese et al. 2013).

Another genome-wide association study in more than 70 000 individuals revealed sortilin-related receptor 1 (SORL1) as a sporadic AD risk gene (Lambert et al. 2013; Rogaeva et al. 2007; Lee et al. 2008). SORL1 is involved in the trafficking and

recycling of APP (Rogaeva et al. 2007) and therefore plays an important role for Aβ generation (Spoelgen et al. 2006; Offe et al. 2006; Schmidt et al. 2007).

Recently, rare missenses mutations in the TREM2 protein have been related to an increased risk of sporadic AD (Guerreiro et al. 2013; Jonsson et al. 2013). TREM2 is a receptor expressed in microglia and regulates phagocytosis and suppresses inflammation (Guerreiro et al. 2013; Rohn 2013). Mutations in TREM2 cause an impairment of microglia phagocytosis thereby reducing the clearance of Aβ. These findings suggest that TREM2 is involved in neurodegeneration associated with the increased risk for sporadic AD.

An interesting finding from a genetic analysis of an Iceland population identified a protective mutation (A673T) in the APP gene (Jonsson et al. 2012). The A673 residue of APP lies very near the BACE1 site. The A673T mutation makes APP less favorable for cleavage by BACE1, resulting in less Aβ production. In addition, the low generation of Aβ peptides lowers Aβ aggregation (Maloney et al. 2014; Benilova et al. 2014). This mutation provides insight that reducing the β-cleavage of APP protects against AD, thus serving to the design of new preventing therapies.

The majority of the identified genes affect Aβ production and clearance, emphasizing the contribution of this pathway in AD pathogenesis. Moreover, the identification of variants related to AD risk provides new insight for understanding the mechanisms underlying AD.

1.5. Mouse models of Alzheimer's disease

In order to study the mechanisms underlying the pathogenesis of AD, many different transgenic animals have been used. From worms, flies, fish to mice and rats, these models mimic the neuropathological alterations of AD, and enable us to study the temporal evolution of AD from its initial stages until the late stages.

Transgenic mice are extensively used in research because their genetic modification is well established and they are easy to bread and house.

The first transgenic mouse model (PDAPP) to study AD expressed high levels of human mutant APP (V717F) developed amyloid plaques between 6-9 months old and exhibit dystrophic neurites (Games et al. 1995). Later, two more mouse models expressing human APP mutations were created: the Tg2576 mouse (expresses APP695 with the Swedish double mutation) (Hsiao et al. 1996) and the APP23

mouse (expresses APP751 with the Swedish double mutation) (Sturchler-Pierrat et al. 1997). These three APP mouse models develop amyloid plaques, dystrophic neurites and gliosis.

Nowadays, there are a variety of transgenic mouse models, which overexpress human APP with mutations associated with the development of FAD. However, mutations in APP are only responsible for a small fraction of FAD cases. Therefore, to represent better the progression of AD, it was necessary to generate mouse models crossing APP mutant lines with lines carrying mutations in the PSEN genes (Holcomb et al. 1998). Double transgenic animals carrying both APP and PSEN1 have increased Aβ42 levels and show development of the disease at young ages (Seabrook and Rosahl 1999). In fact, crossings with PSEN mutations favor the productions of Aβ, in particular Aβ42 peptide (Tara L. Spires and Hyman 2005). However, none of them develop NFTs.

A double transgenic progeny from a cross between the line Tg2576 and the mouse expressing Tau with the P301L mutation, developed the same amount of plaques as the Tg2576 mice but showed enhanced neurofibrillary tangle pathology compared to the P301L mouse (Lewis et al. 2001). A triple transgenic model was created to express APP, PSEN1 and Tau mutations (3xTg), which displays both plaque (from 6 months of age) and tangle pathology (from 12 months of age) (Oddo, Caccamo, Shepherd, et al. 2003; Oddo, Caccamo, Kitazawa, et al. 2003).

In 2006, a new transgenic mouse was generated that coexpresses both APP bearing the Swedish mutation and PSEN1 containing an L166P mutation (APPPS1) (Radde et al. 2006). The Aβ deposition in the APPPS1 mice begins at 6 weeks of age in the cortex and 3-4 months of age in the hippocampus. Amyloid-associated pathologies including dystrophic neurites, hyperphosphorylated tau and robust gliosis, with neocortical microglia number increasing threefold from 1 to 8 months of age (Radde et al. 2006).

Later, another new transgenic mouse model with five FAD mutations (5xFAD) was generated. Although tangles are absent, this model develops an early and severe plaque pathology (around 2 months of age), as well as synapse degeneration (Oakley et al. 2006). Nevertheless, researchers are still not able to generate a mouse model that reproduces all aspects of AD like amyloid plaques, dystrophic neurites, NFTs, neuronal loss, gliosis, synaptic loss and cognitive impairment. And this is the limitation of using a rodent to reproduce a human disease that needs

decades to develop (McGowan, Eriksen, and Hutton 2006). Notwithstanding, the current different transgenic mouse models mimic a range of AD-related pathologies and thus provide significant insights into the pathophysiology of Aβ toxicity and are useful in clinical trials of therapeutic modalities that aim to delay, prevent or stop the disease.

Accordingly to the aim of this study, the AD mouse model used was the APPSwe/PSEN1dE9 (described in chapter 3.1).

2. Amyloid pathology

2.1. Amyloid Precursor Protein

2.1.1 Structure and function

The human APP gene is located on chromosome 21. There are three major isoforms arising from alternative splicing: APP696, APP751 and APP770 (containing 695, 751 and 770 amino acids respectively) (Goate et al. 1991).

APP is evolutionary conserved and detected in lower organisms and in a variety of mammals. The protein family of APP includes APP-like protein 1 and 2 (APLP1 and APLP2) in mammals (Wasco et al. 1992; Wasco et al. 1993; Coulson et al. 2000), APPL in *Drosophila melanogaster* (Rosen et al. 1989) and APL-1 in *Caenorhabditis elegans* (Daigle and Li 1993). All of them are type I transmembrane proteins with a large extracellular N-terminal and short cytoplasmatic C-terminal. The processing of these proteins is in a similar way leading to the secretion of large ectodomains, however only the APP gene contains the Aβ domain (Jacobsen and Iverfeldt 2009).

The biological function of APP remains undetermined, although several studies have been suggesting a role of APP in processes of cellular adhesion, neuronal survival, neurite outgrowth, synaptogenesis, neuronal protein trafficking along the axon, modulation of synaptic plasticity, transmembrane signal transduction, calcium metabolism, etc. (Jacobsen and Iverfeldt 2009; Zheng and Koo 2006).

2.1.2 Proteolytic processing of APP and Aβ generation

APP is synthesized in the endoplasmic reticulum (ER) and then is transported to the trans-Golgi-network (TGN) through the Golgi apparatus (Xu et al. 1997; Hartmann et al. 1997; Greenfield et al. 1999). Generation of Aβ peptides occurs in the ER (for Aβ42) and Golgi/TGN (for Aβ40) (Greenfield et al. 1999).

The proteolytic processing of APP is performed sequentially by three proteinases (α-,β- and γ-secretases) and can be divided in two different pathways: the non-amyloidogenic pathway and the amyloidogenic pathway (Figure 5) (Selkoe 1994a; Selkoe 1994b; Selkoe 1994c; B. De Strooper and Annaert 2000).

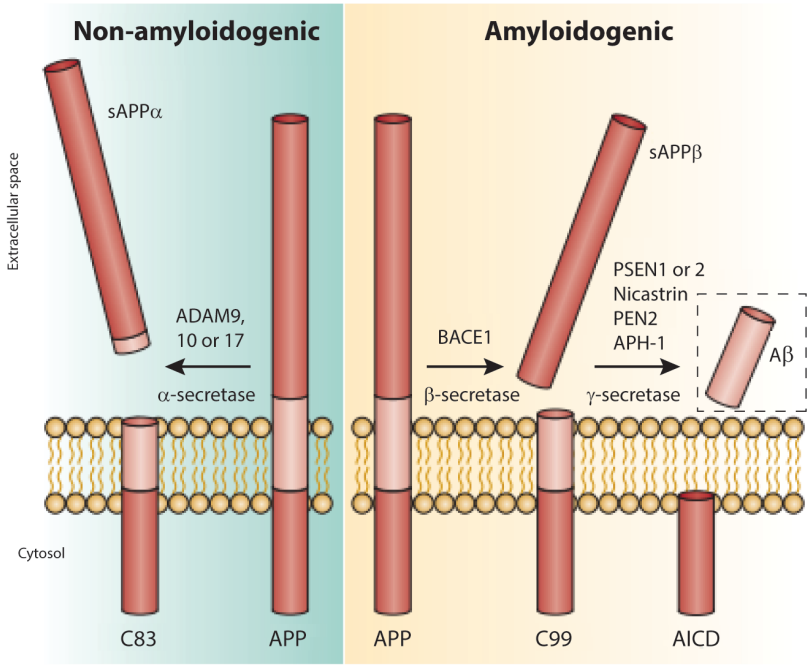


Figura 5: Proteolytic processing of APP within the non-amyloidogenic (left) and amyloidogenic (right) pathways. Figure adapted from (LaFerla, Green, and Oddo 2007).

In the **non-amyloidogenic pathway**, the cleavage of APP is mainly performed by α-secretase that cleaves within the Aβ domain (between the Lys16 and Leu 17) precluding the formation of Aβ (Anderson et al. 1991; Sisodia 1992). This cleavage results in the release of a large soluble ectodomain of APP named sAPPα and the generation of a truncated membrane anchored C-terminal fragment of 83 amino acids named C83 (Sisodia et al. 1990; Esch et al. 1990; Sisodia 1992). The C83 fragment is further processed by γ-secretase generating the small P3 peptide which

apparently is pathologically irrelevant (Haass and Selkoe 1993; Checler 1995). The APP cleavage by α-secretase destroys the Aβ sequence precluding the generation of intact Aβ (Gandy et al. 1994).

In the **amyloidogenic pathway**, the cleavage of APP is firstly performed by β-secretase and results in the generation of Aβ. The β-secretase, known as β-site APP cleaving enzyme 1 (BACE1) (R. Vassar et al. 1999; R. Yan et al. 1999a; Sinha et al. 1999), initiates the cleavage of APP generating a truncated soluble ectodomain, named sAPPβ, and a C-terminal fragment, named C99 (or βCTF), which is retained in the membrane. C99 fragments are further proteolyzed by γ-secretase, which generates two variants of Aβ (Aβ42 and Aβ40) and a residual C-terminal fragment named AICD (APP intracellular domain). The variant Aβ42 has more tendency to aggregate and form fibrils and is also considered more toxic than the shorter Aβ40 (Burdick et al. 1992; Jarrett, Berger, and Lansbury 1993). The α-secretase can compete with β-secretase for the cleavage of APP (Skovronsky et al. 2000) leading to an imbalance between the both cleavage events.

2.1.3 BACE-1

β-secretase activity mediates the initial and rate-limiting factor for Aβ generation. Several studies were undertaken in order to define the characteristics of its activity. The highest levels of β-secretase activity were predominantly found in neurons (Seubert et al. 1993; Zhao et al. 1996) and only membrane-bound subtracts were efficiently cleaved (Citron et al. 1992; Citron, Teplow, and Selkoe 1995). This indicates that β-secretase is likely to be a membrane-bound protease or closely associated with a membrane protein. Additionally, maximal activity of β-secretase was detected at acidic pH, (Haass et al. 1993; Haass, Capell, et al. 1995; Knops et al. 1995), and it was suggested that the active site of this enzyme is located within the lumen of acidic intracellular compartments because the highest β-secretase activity was within the subcellular compartments of the secretory pathway, including the Golgi apparatus and endosomes (Koo and Squazzo 1994; Haass, Lemere, et al. 1995).

Between 1999-2000, five independent groups identified BACE1 (also named as Asp2 or memapsin 2) as the β-secretase because indeed it exhibited all the known

properties of β-secretase (Hussain et al. 1999; Sinha et al. 1999; R. Vassar et al. 1999; R. Yan et al. 1999b; Lin et al. 2000).

BACE1 is a transmembrane aspartic protease composed of 501 amino acids and has a N-terminal signal sequence (residues 1-21), a pro-peptide domain (residues 22-45), a single transmembrane domain near the C-terminus (residues 455-480) and a cytoplasmic tail (Haniu et al. 2000; Benjannet et al. 2001). The correct topological orientation for APP cleavage at the β-secretase site is provided by the location of BACE1 active site within the lumen of intracellular compartments (Haniu et al. 2000).

Besides BACE1, a homologous protease named BACE2 was also identified, however is not involved in Aβ production but can lead to its decrease (Robert Vassar 2004). BACE2 can lower Aβ levels via α-secretase-like cleavage of APP within the Aβ sequence (Hussain et al. 2000; R. Yan et al. 2001; Fluhrer et al. 2002) or work as an Aβ-degrading protease regulating the Aβ levels through its high catalytic efficiency in degrading Aβ intracellularly (Abdul-Hay et al. 2012).

The BACE1 is ubiquitously expressed in the brain and pancreas (R. Vassar et al. 1999). BACE1 levels are elevated in neurons and especially at presynaptic terminals (Zhao et al. 2007; Kandalepas et al. 2013). Elevated expression and activity levels of BACE1 were reported in postmortem brains and cerebrospinal fluid (CSF) from sporadic AD patients (Yang et al. 2003). Accumulation of BACE1 begins to increase in parallel with amyloid pathology and is observed predominantly in dystrophic presynaptic terminals surrounding amyloid plaques in brains of AD patients and AD mouse models (Zhao et al. 2007; Kandalepas et al. 2013) suggesting the strong contribution of BACE1 in the development AD.

BACE1 and APP are separately internalized from the plasma membrane, through different routes, and meet in the early endosomal compartments in order for BACE1 to cleave APP (Rajendran and Annaert 2012; Robert Vassar et al. 2014). Endosomal BACE1 is sorted to lysosomes for its degradation or, alternatively, is transported from early endosomes via Rab11 to recycling endosomes through which it is sorted to the cell surface and then re-internalized back to early endosomes (Rajendran and Annaert 2012). Dysfunctions in the endocytic pathway were observed in brains of sporadic AD patients (A. M. Cataldo et al. 2000; Anne M. Cataldo et al. 2004). Associated with this, an increased endocytic activity enhance the amyloidogenic processing of APP, potentiating the mechanism underlying AD progression (Ginsberg et al. 2010). Moreover, accumulation of APP and the βCTF are

considered the cause of endocytic pathway dysfunction (Jiang et al. 2010; S. Kim et al. 2016). These findings support the hypothesis that altered proteolysis of APP and dysfunction of the endocytic pathway are linked with the abnormal BACE1 activity and increased Aβ production in AD.

Given that BACE1 is the major β-secretase in the brain and is highly contributing to the pathology of AD, a rising number of BACE1 inhibitors have been generated and undergo clinical trials.

2.2. Amyloid peptide

2.2.1. Aggregation and accumulation of Aβ

The Aβ peptide was identified in the mid-80s, for the first time, as component of extracellular amyloid plaques (Glenner and Wong 1984; Masters et al. 1985; Selkoe et al. 1986; Weidemann et al. 1989).

The aggregation of Aβ, especially Aβ42, follows a defined pathway, which results in the formation of amyloid fibrils and plaques. This aggregation starts with Aβ monomers, which aggregate via several intermediate steps and finally form large fibrils with an amyloid structure (Figure 6). Amyloid is defined as “any proteinaceous polymer having a β-pleated sheet conformation that accumulates extracellularly” (Fiala 2007).

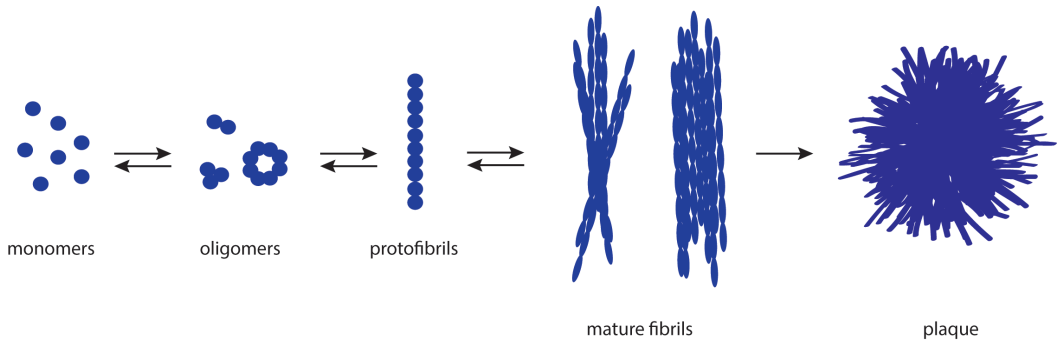


Figure 6: Aβ aggregation process. Aβ is released as a monomer which can aggregate to form oligomers and protofibrils. Protofibrils aggregate to form mature fibrils.

The Aβ peptide is produced as soluble monomers, but quickly aggregates to form multimeric structures. These structures range from dimers and trimers, of low molecular weight (soluble oligomeric species), protofibrils and fibrils of higher molecular weight (insoluble species) that are the basic component of plaques (Cruz et al. 1997; Walsh et al. 1999; Bitan et al. 2003; Ahmed et al. 2010). The fibrillization reaction from toxic protofibrillar intermediates into mature amyloid fibrils can be reversed towards soluble Aβ protofibrils and Aβ monomers (Figure 6) (Martins et al. 2008; Grüning et al. 2013). These dissociations demonstrate that amyloid plaques are the major source of soluble toxic Aβ aggregates.

Amyloid deposition typically occurs via a nucleation-growth mechanism that involves the formation of several intermediates, including soluble oligomers and protofibrils, and has an initial lag-phase due to the thermodynamic unfavorable nucleation event (A. Lomakin et al. 1996; Murphy 2002; Chiti and Dobson 2006). Once nucleation occurs, the aggregation process continues via an exponential growth phase in parallel association with the addition of monomers into aggregate structures. *In vitro* and *in vivo* experiments of Aβ aggregation demonstrated that the initial lag-phase can be circumvented by seeding pre-formed Aβ aggregates which induces and accelerates the process of amyloid plaque deposition (Jarrett and Lansbury 1992; Lansbury 1997; Aleksey Lomakin et al. 1997; Hu et al. 2009; Langer et al. 2011; Hamaguchi et al. 2012).

Insoluble Aβ aggregates appeared to be the most potent inducer for plaque deposition, but others studies revealed that seeding of Aβ oligomers contributed to the plaque formation (Gaspar et al. 2010). Moreover, other *in vitro* and *in vivo* studies showed that Aβ oligomers bind exclusively to synaptic terminals causing alterations of the synapse structure, which leads to synapse loss (Lacor et al. 2007). Supporting the toxic effect of oligomers at the synapse, oligomers induced loss of hippocampal synapses (Brouillette et al. 2012) through modulation of NMDA receptors (Shankar et al. 2007). Synapse loss was associated with reduced dendritic spine density and disruption of memory and learning which might result from enhanced long-term depression mediated by Aβ oligomers (Shankar et al. 2008).

Although compelling evidence suggest that fibrillar aggregation and accumulation of amyloid aggregates might be the leading cause for neurodegeneration in AD, the recent evidences have shift toward defining soluble Aβ oligomers as the toxic agent

rather than plaques bringing up controversies surrounding the amyloid cascade hypothesis.

2.2.2. The amyloid cascade hypothesis

John Hardy and Gerald Higgins firstly described the amyloid cascade hypothesis in 1992 (J. A. Hardy and Higgins 1992). The amyloid cascade combines histopathological and genetic information, and postulates that the deposition of Aβ peptide in the brain parenchyma is the crucial step for the sequence of events that ultimately leads to AD.

According to the hypothesis, Aβ and its multimeric species, triggers a cascade of events producing the pathological characteristics of amyloid plaques and tau aggregates and, ultimately, result in synapse dysfunction and neuronal loss. As Aβ accumulates, the amyloid burden increases and the ongoing neurodegeneration leads to the progression of AD (Figure 7).

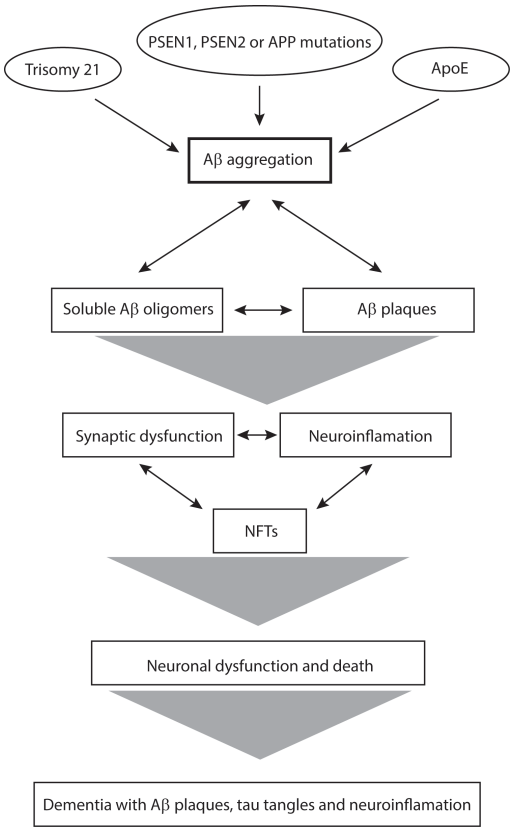


Figure 7: Amyloid cascade hypothesis. Aβ overproduction and/or aggregation trigger the downstream neuronal events and neurodegeneration in AD.

Autosomal dominant mutations that cause early-onset of FAD have supported this hypothesis. These mutations influence the cleavage of Aβ, leading to its accumulation and aggregation into amyloid plaques.

Mutations in the gene encoding for APP or PSEN1 often increase the production of Aβ and/or modify the ratios of Aβ species cleaved from APP. These observations also led to the articulation of the amyloid cascade.

Another putative evidence in support of the hypothesis was the observation of AD like-pathology in individuals with trisomy 21 (Down's Syndrome) (Zigman et al. 2008). Down's syndrome occurs when three copies of the Chromosome 21 are inherited. The gene encoding for APP lies in Chromosome 21, therefore Down's individuals have a triple copy of APP, explaining the excess Aβ production (Zigman et al. 2008).

Although the risk genes mentioned previously contribute for early-onset FAD, a strong genetic risk factor for late-onset of AD with no direct association to the APP gene or its processing enzymes, also support the amyloid hypothesis. This risk factor is the ApoE, which contributes to reduction of Aβ clearance, increasing its levels and aggregation. However, the pathway(s) by which ApoE may increase Aβ levels are still under debate.

The amyloid cascade hypothesis has widely influenced the academia research and the pharmaceutical industry, although some changes to the hypothesis have occurred since recent data suggested soluble Aβ oligomers as the toxic agent rather than plaques (Glabe 2006; Shankar et al. 2007; Bernstein et al. 2009; Kuperstein et al. 2010). Although the hypothesis has been modified, the mechanisms of synaptotoxicity and neurotoxicity mediated by the soluble forms of multimeric Aβ peptide species remain incompletely understood, making this topic controversial in the field. Nevertheless, the theory and the way of interpreting data remains the same, i.e. Aβ production and accumulation as oligomers or fibrillar plaques trigger AD.

2.3. Amyloid plaques

Aβ plaques are one of the central pathologies of AD. Plaques are extracellular deposits of aggregated Aβ, typically within a spherical region, and are abundant in the cortex of AD patients (Fiala 2007). The main components of plaques are the Aβ fibrils, which can be visualized through optical and electronic microscopy (Figure 2).
As Aβ production is enhanced in AD, its accumulation leads to plaque formation. Several groups have been investigating the kinetics of Aβ aggregation into amyloid plaques in AD mouse models (Liebscher and Meyer-Luehmann 2012). These studies revealed that upon Aβ deposition, plaques are very small and then gradually increase their size during ageing (Hyman et al. 1995; P. Yan et al. 2009; Hefendehl et al. 2011; Bittner et al. 2012). Moreover, a new mechanism for plaque growth, called clustering of plaques, proposed that existent plaques contribute to the formation and growth of new plaques in their vicinity (McCarter et al. 2013). Recent *in vivo* data demonstrated that the kinetics of plaque volume is divided in three phases: cubic, transition and saturation. First, the high concentration of Aβ leads to the formation of several new plaques (cubic phase). During the transition phase, the plaque growth rate increases strongly causing an increase of plaque volume. Later, in the saturation phase, plaques grow slower due the high density of plaques. At this stage, as most of the available Aβ is bound in plaques at this stage, there is not enough free available Aβ to promote new plaque formation. Furthermore, not enough Aβ is available to keep up a constant growth rate (Burgold et al. 2014).

2.3.1. Dense-core and diffuse plaques

Amyloid plaques are classified in dense-core and diffuse based on their morphology and Thioflavin-S and Congo Red staining's (Figure 8) (Rak et al. 2007).

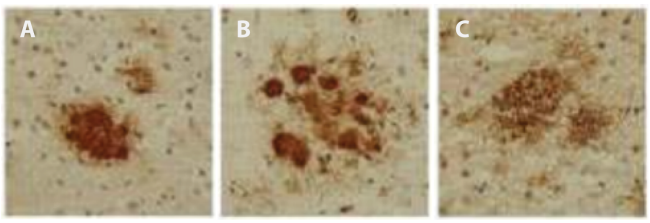


Figure 8: Types of amyloid plaques. (A-B) Dense-core plaques and (B) diffuse plaques immunostained with anti-Aβ antibody (4G8). Figure adapted from (Rak et al. 2007)

Dense-core plaques consist of fibrillar Aβ deposits with a compact core that is well stained with Thioflavin-S and Congo Red. Dense-cored plaques are typically surrounded by dystrophic neurites, activated microglia, reactive astrocytes and associated with synapse loss. They correlate with cognitive impairment in AD patients (Itagaki et al. 1989; Masliah et al. 1994; Pike, Cummings, and Cotman 1995; Knowles et al. 1999; Urbanc et al. 2002; Sasaki et al. 2002; Vehmas et al. 2003).

Diffuse plaques consist of amorphous Aβ (Tagliavini et al. 1988; Yamaguchi et al. 1989) and are proposed to be the precursors of dense-cored plaques (Armstrong 1998). These plaque types are generally non-neuritic and not associated with synapse loss. Thereby, they are common found in brains of cognitively intact aged people (Mirra 1997; Rak et al. 2007; A. Serrano-Pozo et al. 2011). Contrary to dense-core plaques, these amyloid deposits are Thioflavin S and Congo Red negative.

Structurally, dense-core and diffuse plaques are very different. As verified by an anti-Aβ antibody therapy, APP transgenic mice treated with the antibody showed a reduced plaque load with preferential clearance of diffuse plaques (A. Wang et al. 2011).

Only dense-core plaques and not diffuse plaques are associated with neuritic and inflammatory pathology in AD patients as well as in AD mouse models. However, transgenic mouse models expressing FAD mutations develop different types of amyloid plaques. APP23 mice mice developed both Congo Red positive dense-core plaques and diffuse plaques (Sturchler-Pierrat et al. 1997; Stalder et al. 1999) while Tg2576, PSAPP and APPPS1 mainly developed dense-core plaques (Sasaki et al. 2002; Kumar-Singh et al. 2005; Pereson et al. 2009).

Some AD patients with PSENΔE9 develop atypical neuropathology for AD, including large “cotton wool” amyloid deposits in the cortex with a relative absence of dense-core plaques (Crook et al. 1998; Verkkoniemi et al. 2001). “Cotton wool” plaques are large, ball-like plaques lacking dense amyloid cores that displace adjacent structures. However, APPSwe/PSEN1dE9 mice do not display “cotton wool” plaques but have the typical AD dense-core plaques (Jankowsky et al. 2004).

The type of plaque is important for the pathological diagnosis of AD because it is a relatively common to find diffuse plaques the brain of cognitively intact elderly people. New insights into the mechanisms that lead to the formation of different

amyloid plaques would contribute to a better understanding of pathological AD progression.

2.3.2. Plaque-associated axonal dystrophies

Plaque-associated neuritic dystrophies (Figure 9) are one of the diagnostic brain lesions observed in AD patients and represent the most notorious evidence that Aβ might induce neurotoxicity. These axonal and dendritic injuries, generally found in large numbers in the cortex, causing a permanent disruption of neuronal connections (D. W. Dickson 1997; Vickers et al. 1996; J. Hardy and Selkoe 2002; Tsai et al. 2004; Bittner et al. 2012).

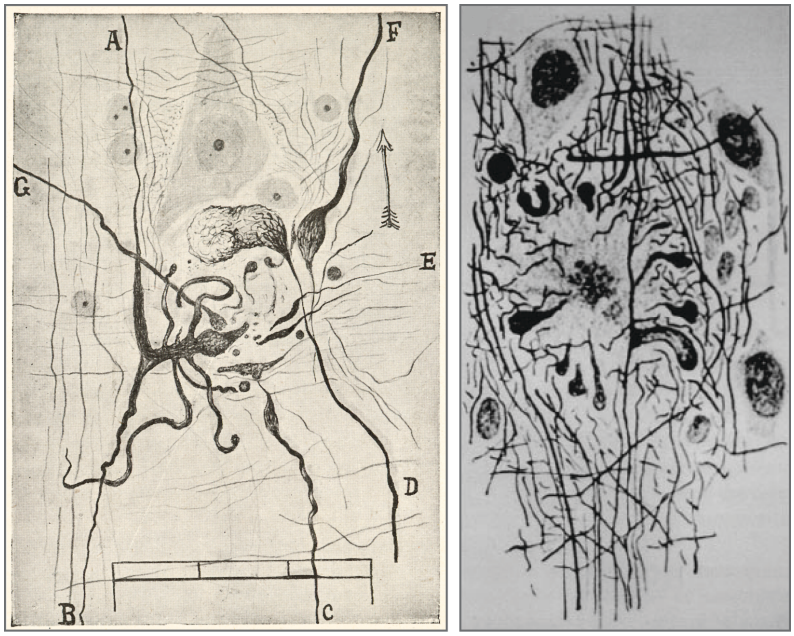


Figure 9: Illustration of dystrophic neurites surrounding an amyloid plaque. Drawings from Simarro (left) and Oskar Fischer (right). Figure adapted from (Cajal and May 1991).

Dystrophic neurites are often swollen and tortuous axons (axonal dystrophies), with variable morphology and composition depending on the pathological stage of AD (Vickers et al. 1996; Su, Cummings, and Cotman 1998; Knowles et al. 1999; Woodhouse et al. 2009; Wu et al. 2010; Mitew et al. 2013). Axonal dystrophies are intimately associated with extracellular deposits of Aβ, principally amyloid fibrils (Geddes, Anderson, and Cotman 1986; Masliah et al. 1991; Masliah et al. 1994;

Vickers et al. 1996; Su, Cummings, and Cotman 1998; T. C. Dickson et al. 1999; D'Amore et al. 2003; R. Brendza 2003; Tsai et al. 2004; T. L. Spires 2005; Monica Garcia-Alloza, Dodwell, et al. 2006; Woodhouse et al. 2009). They can occur both within the amyloid plaque and/or immediately surrounding it.

The accumulation of Aβ in the brain of AD patients results from the proteolytic processing of APP by BACE1 and γ-secretase (Bart De Strooper 2010). The role of BACE1 in AD came from the analysis of postmortem brains and CSF from AD brains where BACE1 levels and activity are increased (Fukumoto et al. 2002; Holsinger et al. 2002; Tyler et al. 2002; Yang et al. 2003; Li et al. 2004; Harada et al. 2006). Elevation of BACE1 is not uniform throughout the brain, but is predominantly concentrated in dystrophic presynaptic terminals surrounding amyloid plaques (Zhao et al. 2007; Kandalepas et al. 2013). BACE1 and APP strongly accumulate in dystrophies (Cras et al. 1991; Kandalepas et al. 2013) and potentiates the generation of BACE1-cleaved APP products, including Aβ42 that exacerbates plaque growth (Sadleir et al. 2015; Sadleir et al. 2016).

Although most of the previous studies have focused on the analysis of the localization pattern of increased BACE1 in dystrophic axons surrounding plaques, the mechanisms for axonal dystrophy formation remained unclear until recently. Two comprehensive studies provided new insights into the fundamental mechanisms for axonal dystrophy formation, Aβ generation and plaque growth. Importantly, they revealed that the formation of axonal dystrophies is caused by a feed-forward mechanism of increased accumulation of BACE1, APP and lysosomes (preferentially lysosome precursors) as a result of a local impairment in the retrograde axonal transport. Accumulation of immature lysosomes at amyloid plaques acts as sites of Aβ synthesis increasing its accumulation. The increase of Aβ generation and plaque growth cause axonal microtubule disruption and microtubule-based transport impairment leading to the accumulation of BACE1, APP and Aβ generation (Gowrishankar et al. 2015; Sadleir et al. 2016).

3. Investigating plaque-associated presynaptic dystrophies

Previous studies have reported *in vivo* evidence for the formation of axonal dystrophies near plaques and the possibility of recovery after different treatments (M. Garcia-Alloza et al. 2007; R. P. Brendza et al. 2005; T. L. Spires 2005; Tsai et al. 2004; D'Amore et al. 2003). However, these studies did not monitor the formation of axonal dystrophies and plaque growth kinetics over a long observation period including a long-term therapeutic strategy.

3.1. The APPSwe/PSEN1dE9 mouse model

Transgenic mouse models recapitulate some of the pathological aspects of AD allowing the analysis of disease initiation and progression.

The APPSwe/PSEN1dE9 mouse model is double transgenic mouse that was created by breeding mice that express mutant APP with mice that express mutant PSEN1.

The Swedish mutation (APPSwe) causes an increase of the total Aβ generation (Borchelt et al. 1996; Savonenko et al. 2005). Expressing human PSEN1 lacking exon 9 mimics the FAD-associated mutation. Plaque depositions in APPSwe/PSEN1dE9 mice form at six months of age with an even greater amyloid burden at 18 months (Savonenko et al. 2005).

Although some AD patients with ΔE9 develop atypical neuropathology for AD, APPSwe/PSEN1dE9 mice display typical dense-core plaques in the cortex and develop axonal dystrophies surrounding plaques (Jankowsky et al. 2004; Kitazawa, Medeiros, and LaFerla 2012).

The generation of a new mouse model which axons, dendrites and dendritic spines express GFP-M (Feng et al. 2000) enabled us to generate the mouse model used in this study (APPSwe/PSEN1dE9 x GFP-M). The APPSwe/PSEN1dE9 crossed with the GFP-M mouse line leads to a sparse labeling of pyramidal neurons in neocortex and hippocampus (Feng et al. 2000) allowing us to study the formation of axonal dystrophies associated with plaque development over time. Given that this mouse model develop plaques later than the APPPS1 mouse model (Radde et al. 2006) it recapitulates the gradual progression of AD, which makes a good model to study plaque-associated axonal dystrophies.

3.2. *In vivo* two-photon imaging in mouse models of AD

BACE1 is the major β-secretase enzyme required for the generation of Aβ peptides. Therefore, has emerged as a promising target for the treatment of AD. To investigate the pharmacological effect of BACE1 inhibition on the formation of axonal dystrophies and plaque development, long-term *in vivo* 2-photon imaging was used to obtain the majority of the results for this thesis. The 2-photon microscopy allowed to image single axons and monitors the formation of axonal dystrophies and plaque development before and after BACE1 inhibitor administration, in the same mouse model, over a long period of time. Combining 2-photon microscopy with a therapeutic strategy is important to interpret and characterize the effect of BACE1 inhibition in a mouse model of AD. By implanting a cranial window over the somatosensory cortex and applying (Figure 10) 2-photon microscopy (Holtmaat et al. 2009) it is possible to perform long-term imaging of neuritic structures and study the dynamics of cortical pathology at a far greater imaging depth compared to conventional confocal microscopy. Two-photon microscopy uses pulsed infrared light with a long wavelength to excite fluorophores preventing the effect of tissue light scattering at greater depths. The fluorophores absorb two photons nearly simultaneously, instead of the one in conventional microscopy. The non-linear effect of 2-photon excitation diminishes the area of out of focus-excitation compared with conventional fluorescence microscopy, therefore reducing out of focus excitation and photobleaching.

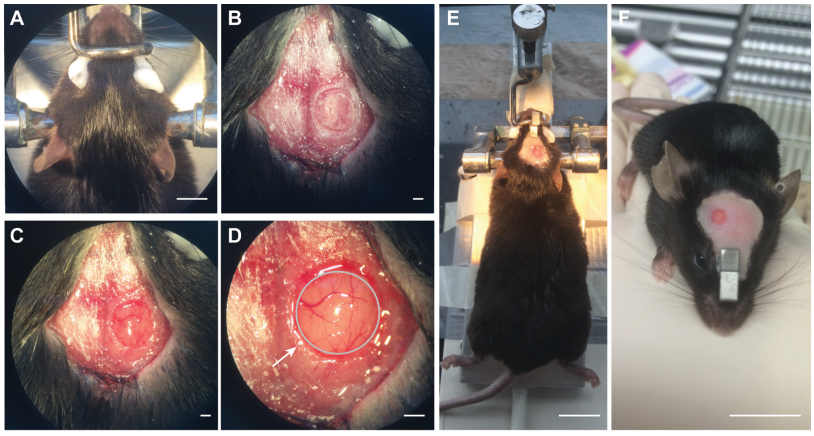


Figure 10: Images of a 4-mm cranial window implantation over the somatosensory cortex. (A) Head fixation. (B) Trepanation of the skull. (C) Image of the cranial window after the piece of the skull has been removed and brain after the cleaning procedure. (D) 4-mm cranial window. (E) Image of the cranial window after the application of dental cement over the skull. (F) Cranial window four weeks after surgery. *Scale bars*: 1 mm and 20 mm.

Fluorescent dyes such as Methoxy-X04 cross the blood brain barrier and can be repeatedly administrated via peripheral injection. Methoxy-X04 labels amyloid fibers allowing the visualization of individual plaques over time (Klunk et al. 2002; Hefendehl et al. 2011).

Generation of mice that express eGFP in neurons enabled the visualization of neurites and dendritic spines using 2-photon microscopy and the study of the relationship between amyloid plaques and associated pathology (Monica Garcia-Alloza, Dodwell, et al. 2006; Meyer-Luehmann et al. 2008; Bittner et al. 2012).

Despite the limited imaging area, 2-photon microscopy is a powerful tool to image the dynamics of axonal dystrophies and the kinetics of amyloid plaques during a long period of time.

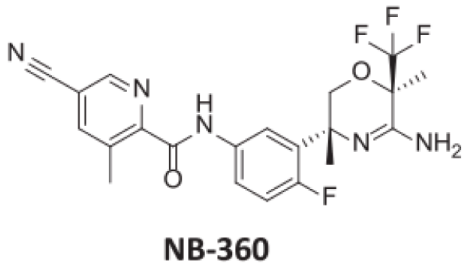
3.3. BACE1 inhibitor – a prime drug target for AD

Previous studies have reported *in vivo*, evidences about the possibility for recovery of the formation of axonal dystrophies near plaques after different treatments. (M. Garcia-Alloza et al. 2007; R. P. Brendza et al. 2005; T. L. Spires 2005; Tsai et al. 2004; D'Amore et al. 2003).

Given that BACE1 is the required initiator enzyme for Aβ generation and therefore rate-limiting in the production of Aβ (R. Vassar et al. 1999; Ghosh, Brindisi, and Tang 2012), it is one of the major drug targets for the treatment of AD.

In BACE1-null mice, Aβ generation is abolished, and Aβ is significantly reduced in BACE1 heterozygous mice (McConlogue et al. 2007). Based on these data, several pharmaceutical companies have developed potent blood brain barrier (BBB)-penetrant BACE1 inhibitors that are being tested at various stages of clinical trials with the ambition that potent inhibition of BACE1 activity in humans will decrease Aβ generation, benefit synaptic function and stop the progression of the disease (Riqiang Yan and Vassar 2014).

In this study we used the novel BACE1 inhibitor – NB360, described in 2015 by (Neumann et al. 2015) (Figure 11). NB360 is a potent and BBB-penetrant BACE1 inhibitor which greatly reduced Aβ and neuroinflammation in rodent and non-rodent models (Neumann et al. 2015).



NB-360

Figure 11: Structure of the BACE1 inhibitor NB360. NB360 is orally administrated by food pellets (Neumann et al. 2015).

MATERIAL AND METHODS

1. Genotyping

A small section of tail was removed from each mouse for genotyping. First, the DNA was extracted following instructions of the Invisorb® DNA Tissue HTS 96 Kit/C (Stratec molecular). The tissue was incubated in 400 µl Lysis Buffer G (inclusive Proteinase K) at 52°C under continuously shaking until lysis is completed and centrifuge at 1.700 x g (4.000 rpm) for 10 min and RT. Supernatant was carefully transferred into a 2 ml Collection Plate and 200 µl Binding Buffer A were added to each well of the 2 ml Collection Plate and mix it by pipetting up and down. The plate was centrifuged at 1.700 x g (4.000 rpm) for 5 min at RT. The filtrated was discarded and the plate air-dried. The pellet was re-suspended in 550 µl of Wash Buffer and centrifuged at 1.700 x g (4.000 rpm) for 5 min at RT followed by another centrifugation for at least 15 min at max. 1.700 x g (4.000 rpm). To finalize the DNA extraction, 100 µl prewarmed elution buffer (52°C) was added in each well and centrifuged for 5 min at 1.700 x g (4.000 rpm). The extracted DNA was subjected to a polymerase chain reaction (PCR) to amplify PSEN1 gene - if present. The PCR solution consisted of: 12,5 µl OneTaq HotStart QuickLoad, 0,5 µl of each forward primer (CTA GGC CAC AGA ATT GAA AGA TCT; AAT AGA GAA CGG CAG GA), 0.5 µl of each reverse primer (GTA GGT GGA AAT TCT AGC ATC ATC C; GCC ATG AGG GCA CTA AT), 0,5 µl template DNA and 10 µl distilled water. This solution was placed in a thermocycler andthe following PCR program was used:

Step	Temperature (°C)	Time	Repeat
1	94	3 minutes	1x
2	94	30 seconds	27x
3	54	1 minute	
4	68	40 seconds	
5	68	5 minutes	1x
6	10	1 min	

Another PCR to amplify eGFP gene - if present, consisted of: 12,5 µl OneTaq HotStart QuickLoad, 0,5 µl of forward primer (AAG TTC ATC TGC ACC ACC G), 0.5 µl of reverse primer (TCC TTG AAG AAG ATG GTG CG), 0,5 µl template DNA and

11 µl distilled water. This solution was placed in a thermocycler. The following PCR program was used:

Step	Temperature (°C)	Time	Repeat
1	94	3 minutes	1x
2	94	30 seconds	27x
3	60	1 minute	
4	68	20 seconds	
5	68	2 minutes	1x
6	10	1 min	

The PCR samples were separated by gel electrophoresis using a 1,5% agarose containing SYBR® Gold Nucleic Acid Gel Stain in TAE buffer as the running buffer. Between 120-195 V was applied for approximately 60-90 minutes and the gel imaged with a UV light source. A photograph was taken for documentation.

2. Animals and Housing

The studies were carried out in accordance with an animal protocol approved by the Ludwig-Maximilians-University Munich and the government of Upper Bavaria (Az. 55.2-1-54-2532-62-12). The cranial window preparation and *in vivo* imaging were performed under anesthesia, and all efforts were made to minimize suffering of the animals.

Mouse lines APPSwe/PSEN1dE9 (Jankowsky et al. 2004) and GFP-M (Feng et al. 2000) were used in this study. The double transgenic APPSwe/PSEN1dE9 mouse line expresses a chimeric protein of mouse/human amyloid precursor (Mo/HuAPP695swe) and mutant human presenilin 1 (PSEN1-dE9) both under the control of the mouse prion protein promoter resulting in abundant amyloid plaques in neocortex and hippocampus starting at the age of 6 months (Jankowsky et al. 2004; Savonenko et al. 2005). In the GFP-M mouse line the green fluorescent protein (eGFP) is expressed under control of Thy1.2 promoter which leads to a sparse labeling of pyramidal neurons in neocortex and hippocampus (Feng et al. 2000). Heterozygous mice of APPSwe/PSEN1dE9 were crossed with heterozygous GFP-M mice resulting in triple transgenic APPSwe/PSEN1dE9 x GFP-M offspring, which were inbred. Heterozygous triple transgenic mice of mixed gender were used for experiments at indicated ages described below. Mice were group-housed under

pathogen-free conditions until surgery, after which they were singly housed in standard cages with food and water *ad libitum*.

3. BACE1 Inhibitor

NB360 was synthesized following the schemes provided by Novartis (Neumann et al. 2015) and formulated in 10% (w/v) 2-hydroxypropyl-beta-cyclodextrin. Both inhibitor and vehicle were administered orally via the food pellets at 0.25 g/kg body weight. Assuming 30g body weight and 4 to 5 g daily food consumption, the mice received a daily dose of 33 to 42 mg/kg body weight for up to 3 months. The pharmacological properties of NB360 have been extensively characterized previously (Neumann et al. 2015).

4. Two-Photon *in vivo* imaging

For *in vivo* imaging, a chronic cranial window was implanted as described previously (Fuhrmann et al. 2007; Holtmaat et al. 2009). Surgery was performed in eight 5 months-old APPSwe/PSEN1dE9 x GFP-M. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (14 mg/Kg body weight; WDT/Bayer Health Care). Additionally, dexamethasone (6 mg/kg body weight; Sigma) was intraperitoneally administered immediately before surgery. Utilizing the open-skull preparation, a cranial window was placed above the somatosensory cortex. For repositioning during repetitive imaging a small titan bar was glued next to the window. After surgery, mice received subcutaneously analgesic treatment with carprophen (7.5 mg/Kg body weight; Pfizer) and antibiotic treatment with cefotaxim (250 mg/Kg body weight; Pharmore).

Weekly imaging started 4 weeks post-surgery (recovery period) utilizing a LSM 7 MP setup (Zeiss) being equipped with a MaiTai laser (Spectra Physics) followed by imaging under treatment with NB360 or vehicle food pellet for 3 months. Around 24 hours before imaging, Methoxy-X04 (0.4 to 2.4 mg/Kg body weight, Xcessbio, San Diego, CA, USA) was intraperitoneally injected to stain *in vivo* amyloid plaques (Klunk et al., 2002.). Mice were anesthetized by isoflurane supply during each *in vivo* imaging session. Two-photon excitation of Methoxy-X04 labelled Aβ plaques was performed at 750 nm and the signal was detected using a short pass (SP) 485 nm

filter. Two-photon excitation of eGFP-expressing neuronal structures was performed at 880 nm and the signal was detected using a bandpass (BP) 500-550 nm filter. To exclude autofluorescent spots from analysis, we also recorded emission signals at 590-650 nm. These auto-fluorescent spots were found in the neuropil and within neuronal and glial cells. A x20 1.0 NA water-immersion objective (Zeiss) was used. Overview images of eGFP expressing neuronal structures were taken at low resolution (logical size 512 × 512 pixels; physical size x, y, z: 424.3 x 424.3 x 300 µm; z-step = 3 µm) up to a depth of 300 µm to find the same position over time. Overview images of amyloid plaques were taken at high resolution (1300 x 1300 pixels; physical size x, y, z: 424.77 x 424.77 x 200.00 µm). At least 2-3 overviews were taken per animal at each imaging session. High-resolution images (logical size 512 × 512 pixels; physical size x, y, z: 84.9 x 84.9 x 40-60 µm; z-step = 1 µm) of single Aβ plaques and eGFP-expressing axons/neurites surrounding them were taken for further analysis. Dystrophic axons were 3D reconstructed over time.

5. Immunohistochemistry and confocal imaging

At the end of the long-term 2-photon imaging, mice were anaesthetized with an intraperitoneal injection of a solution of ketamine/xylazine (0.14 mg / g body weight; WDT / Bayer Health Care) and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.12 M sodium phosphate buffer (PB), pH: 7.4. The brain was then removed and post-fixed for 24 h at 4°C in the same fixative. To obtain coronal sections, the brain was washed in 0.1 M PB and sections of 50 µm were cut with a vibratome (Leica VT 1000S). The study was focused on the supragranular neocortical layers between bregma −1.64 and −2.12 mm, with the aim of analyzing similar layers and cortical areas as in the *in vivo* study. In addition, six 10 months-old mice which were not used for *in vivo* imaging experiments were kept under treatment (NB360 or vehicle food pellets) as described before.

Immunohistochemistry was performed on free-floating sections to perform a quantitative analysis of the presence of different markers in eGFP-expressing dystrophic axons. Samples were permeabilized (2% Triton-PBS) overnight at room temperature. Sections were blocked (10% normal goat serum-PBS for 2h at room temperature, followed by incubation with primary antibody (diluted in 3% normal goat

serum, 0.3% Triton-X100, 0.05% sodium azide – PBS) for 2 overnights at 4°C. Sections were washed in 3% normal goat serum, 0.3% Triton-X100, 0.05% sodium azide – PBS (3x10 min) and then labeled with fluorescently labeled secondary antibodies, diluted in the same buffer as for first antibodies, overnight at 4°C. Sections were washed again (3x10 min). Fibrillar Aβ plaques were stained with Methoxy-X04 (0.01 mg/ml in 0.1 M PB) for 20 min at room temperature and washed (3x10 min). Sections were then mounted on microscope slides using Fluorescent Mounting Medium (Dako, Glostrup, Denmark).
Antibody information (including commercial sources and dilutions) can be found in Table 1.

Table 1: List of antibodies used for immunostainings.

Antibody	Source	Catalog/clone no.	Dilution
LAMP1	Abcam	ab25245	1:500
BACE1	Cell Signaling	5606S	1:200
Aβ42	Millipore	ABN 13	1:400
Aβ4G8	BioLegend	SIG-39220	1:500

To analyze normal and dystrophic axons around Aβ plaques, 16-bit-images (logical size 2048 x 2048 pixels; physical size x, y, z: 212.44 x 212.44 x 29.50 µm and logical size 1024 x 1024 pixels; physical size x, y, z: 106.17 x 106.17 x 20.80 µm) were acquired with a LSM 780 confocal microscope (Zeiss) equipped with a laser and with the objective Plan Apochromat 40x 1.4 Oil DIC M27. For analysis of the number of plaques containing dystrophic and normal axons after both NB360 and vehicle treatment, fluorescence images of somatosensory cortex were acquired through the 20x objective (Zeiss Plan-NEOFLURAR) in frames, using Apotome 2 microscope (Carl Zeiss).

6. Aβ quantification

Forebrains from APPSwe/PSEN1dE9 x GFP-M mice were isolated at the end of treatment period, frozen and homogenized in 9 volumes of ice-cold Tris-buffered

saline (pH 7.4) containing Complete protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany) using a Sonifier 450 (Branson) and stored in aliquots at -80 °C. Triton X-100 soluble Aβ was extracted by mixing 50 µl 2 % Triton X-100 with 50 µl homogenate, incubated for 15 min on ice and subsequently vortexed, followed by ultracentrifugation at 100.000xg for 15 min. The clear supernatant was diluted to a final forebrain dilution of 1:100 and used for analysis. For the extraction of insoluble amyloid peptides, 50 µl forebrain homogenate was mixed with 117µl of 100 % formic acid and stored on ice for 15 min with vortexing. Samples were neutralized with 950 µl 1 M Tris base, containing Complete protease inhibitor cocktail (Roche, Basle, Switzerland) and stored overnight at room temperature. After 15 min centrifugation at 14000 rpm the supernatant was used for analysis.

Aβ40 and 42 were determined using the electrochemiluminescence immuno assay kits from Meso Scale Discovery (Rockville, MD, USA) in either singlet or triplex format. Samples and standards were prepared according to the manufactures protocols. The kit based on 6E10 was used for CSF or plasma samples and APPSwe/PSEN1dE9 x GFP-M brain. Soluble APPα and sAPPβ from mice brains were determined from the 100.000xg supernatant and analyzed with Meso Scale Discovery commercial kits. C-terminal fragments C83 and C99 were determined from Western blots. Forebrain homogenates dephosphorylated with lambda protein phosphatase and run on a 10 % Tris-bicine gel with 8 M urea. After transfer to Immobilon P membranes (Bio-Rad Bedford MA USA), bands were probed with APP C8 antibody (recognizing the C-terminus of APP) and detected with goat anti-mouse IgG Fab fragment AlexaFluor680 (Invitrogen). The same gel was used to visualize Aβ1-40 and 1-42 using the N-terminal antibody beta1 and goat anti rabbit IgG IRdye 800CW (Odyssey). Full-length human APP was detected using an in house immune assay based on the MSDECL system.

7. Electron microscopy preparation and TEM imaging

Three APPSwe/PSEN1dE9 mice vehicle-treated and three APPSwe/PSEN1dE9 mice NB360-treated were transcardially perfused with 4% paraformaldehyde in 0.12 M PB, pH 7.4. Plastic-embedded sections were studied by correlative light and electron microscopy, as described in detail elsewhere (J. DeFelipe and Fairén 1993). Briefly, sections were photographed under the light microscope and then serially cut

into semithin (2-µm thick) sections on a Leica ultramicrotome (EM UC6, Leica Microsystems). The semithin sections were stained with 1% toluidine blue in 1% borax, examined under the light microscope, and then photographed to locate Abeta plaque regions of interest. Serial ultrathin sections (50- to 70-nm thick) were obtained from selected semithin sections on a Leica ultramicrotome, and collected on formvar-coated single-slot nickel grids and stained with uranyl acetate and lead citrate. Digital images were captured at different magnifications on a Jeol JEM-1011 TEM (JEOL Inc., MA, USA) equipped with an 11 Megapixel Gatan Orius CCD digital camera.

8. Images, data processing and statistics

Deconvoluted 2-photon images (AutoQuantX2, Media Cybernetics) were processed by Imaris software (Bitplane AG, Zurich, Switzerland) to obtain the 3D reconstructions of the dystrophic axons and Aβ plaques for analysis of the volumes of each of them at the different time points. Plaque volumes were extracted by 3D-surface-rendering with background subtraction and a threshold of 500.

Dystrophic axons were manually segmented in the image stacks. Only dystrophic and parent axons that were present in the whole imaging stack in all time points were reconstructed. An axonal segment was considered dystrophic when its volume doubled the volume of the non-dystrophic axonal segment.

In order to better show the details of the dystrophic axons, the representative images were processed using the burn tool in Adobe Photoshop CS6 to reduce the autofluorescence.

Data are represented as mean±SD unless specified otherwise. Data were analyzed by using GraphPad Prism 5.04 software (GraphPad Inc., La Jolla, CA, USA) using the indicated statistical tests.

RESULTS

1. Reduction of Aβ40 and Aβ42 levels by pharmacological inhibition of BACE1

The novel BACE1 inhibitor NB360 (Figure 11) can be administrated orally (food pellet dosing, 0.25 g/kg). It crosses effectively the BBB and has been shown to decrease the progression of Aβ deposition in the brains of APP transgenic mice as well as a reduction of Aβ40 and Aβ42 levels in different species like rats, dogs and mice (Neumann et al. 2015). We verified these findings in our mouse model, in which treatment for 15 days with NB360 caused a significant decrease in Aβ40 and Aβ42 levels in cerebral cortex and plasma (Figure 12). Vehicle-treated mice did not show significant changes of Aβ40 and Aβ42 levels either in cortex or plasma (Figure 12).

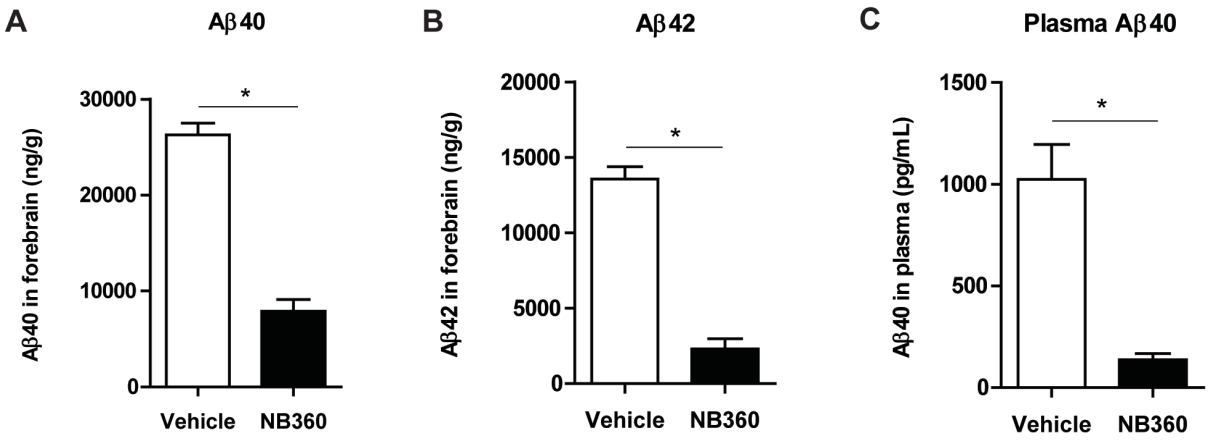


Figure 12: Pharmacological inhibition of BACE1 potently reduces Aβ40 and Aβ42 levels. The Aβ40 and Aβ42 levels are reduced in mouse cortex (A, B) and the Aβ40 levels are reduced in plasma (C) by NB360 after 15 days of administration through food pellets. Data presented as mean ± SEM, n = 4 per group; t-test. *p < 0.05; **p < 0.01; ***p < 0.001.

2. Inhibition of BACE1 induces hair depigmentation

A recent publication on long-term effects of BACE1 inhibitor on hair depigmentation showed that chronic dual exposure to the equipotent BACE1/BACE2 inhibitor (NB360) displayed a dose- and exposure- dependent and irreversible hair depigmentation due to the inhibition of BACE2 which blocks PMEL17 processing and reduces melanin production (Shimshek et al. 2016). Indeed, in our study, 2-3 weeks

after initialization of BACE1 inhibitor treatment mice showed hair depigmentation. In some animals the hair depigmentation was on the ventral part and later was spread both on ventral body and dorsal part with an irregular pattern (Figure 13). Vehicle-treated mice did not show any hair color changes.

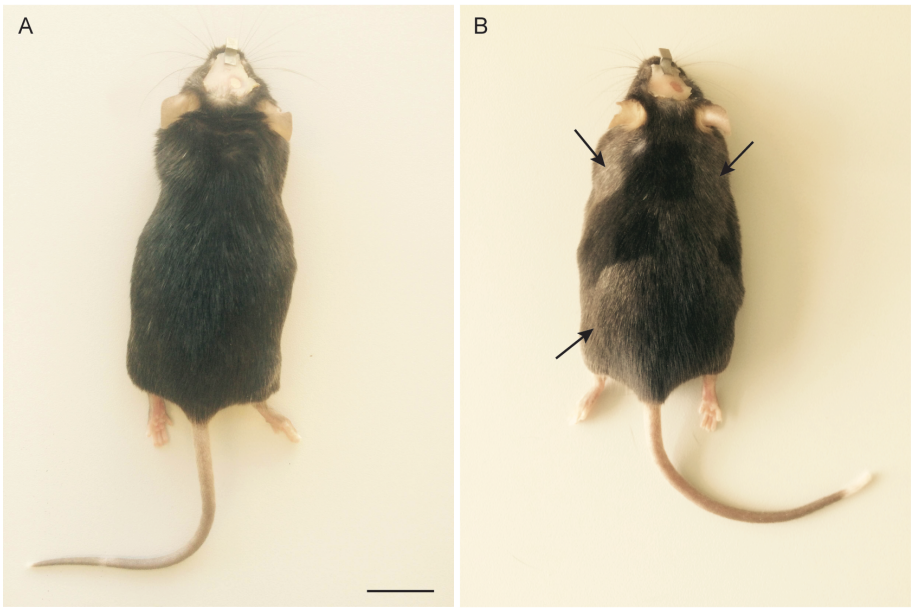


Figure 13: BACE1 inhibition induces hair depigmentation in mice. (A) Example photo of vehicle-treated mouse. (B) Upon chronic treatment with NB360 mice developed grey patches (black arrows) on their fur. *Scale bar* 20 mm.

3. BACE1 inhibitor rescues the dynamics of axonal dystrophies

Several approaches to target Aβ production via inhibition of γ-secretase or antibody therapy have not yet resulted in considerable clinical benefits. Besides, the persistent challenging concept of designing BACE1 inhibitors, which can efficiently penetrate the brain and decrease Aβ levels, has proven their potential therapeutic approach for limiting plaque-associated presynaptic pathogenesis. However, due to some failures with clinical trials of BACE1 inhibitors, new compounds are currently under studies to test their proper efficacy and safety profiles (Lucas, Fukushima, and Nozaki 2012; May et al. 2011; H. Wang, Li, and Shen 2013; Yuan et al. 2013; Riqiang Yan and Vassar 2014; Robert Vassar and Kandalepas 2011).

To study possible benefits of long-term BACE1 inhibition on the dynamics of plaque-associated axonal dystrophies, we performed chronic *in vivo* 2-photon imaging in the somatosensory cortex of adult APPSwe/PSEN1dE9 x GFP-M mice. One month after

the implantation of the cranial window, we identified in the supragranular layers of the somatosensory cortex axonal dystrophies, which were visible close to Aβ plaques (Figure 14). The imaging started when mice were 6 months-old (initial stage of amyloid pathology) and was prolonged until the age of 10 months-old (advanced stage of the disease).

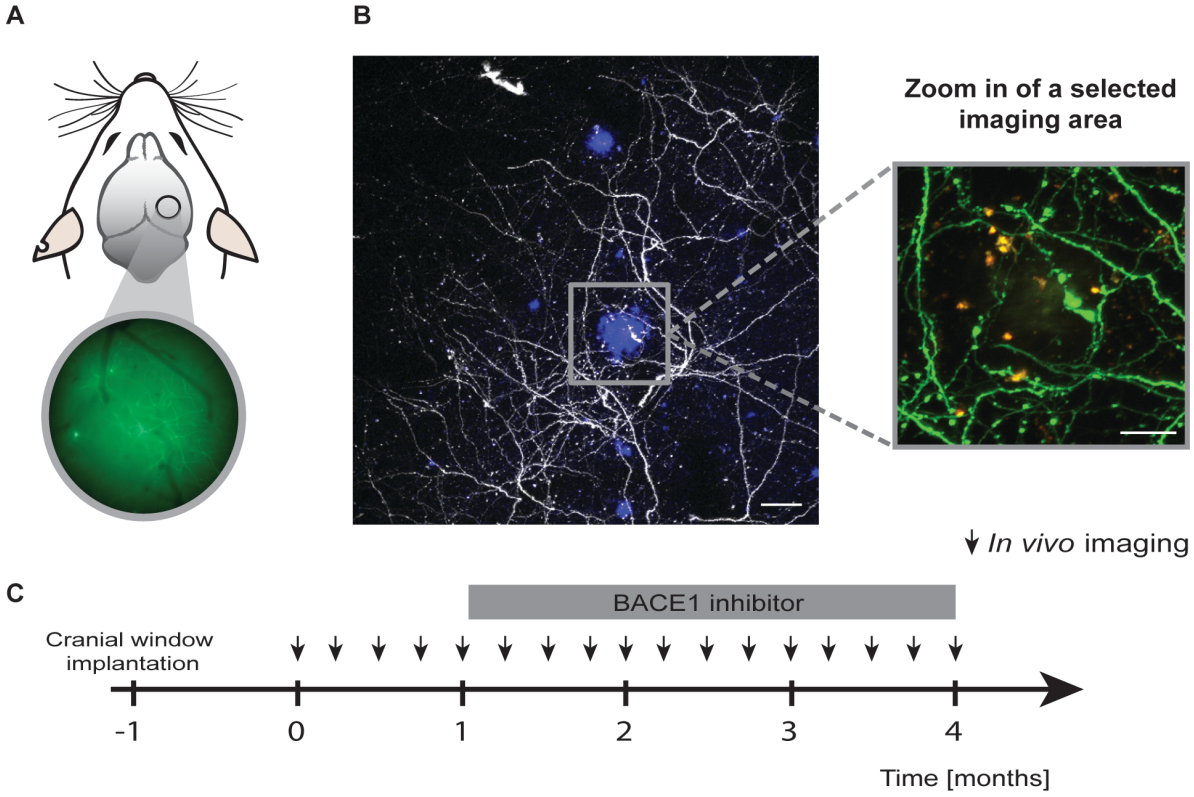


Figure 14: Dystrophic axons in the supragranular layers of the somatosensory cortex and long-term *in vivo* imaging of APPSwe/PSEN1dE9 x GFP-M mice. (A) Schematic representation of the cranial window implanted over the somatosensory cortex. (B) Overview and detailed projection of the selected imaging area. (C) Experimental timeline: 4 weeks after window implantation (month zero) imaging was performed over four months, once every week (black arrowheads) and after the first month of imaging, animals were treated with BACE1 inhibitor or vehicle. *Scale bar* 20 µm.

The individual study of the axons and their dystrophies was achievable due to the crossing between our AD model and the GFP-M model, which displays a low density of neurons expressing eGFP.

The dynamics of axonal dystrophies were monitored before and during the treatment with NB360 (Neumann et al. 2015) (food pellet dosing 0.25g/kg body weight; over three months) and compared with vehicle-treated animals (Figure 15A-C).

Using the Imaris software, all observed dystrophic axons were 3D reconstructed (Figure 15 – lower panels of each representative image) and the volume of each dystrophy was measured in order to study their variation over time.

Interestingly, three weeks after treatment with BACE1 inhibitor, the volume of the axonal dystrophies decreased significantly (Figure 16B). During the treatment, we observed a gradual and notably reduction of the volume in the majority of detected dystrophies (Figure 16B and C), an effect that was paralleled with the absence of development of new dystrophies. In vehicle-treated mice, we observed a dynamic variability in the volume of the axonal dystrophies over time (Figure 16A and C).

Overall, our data demonstrate a significant effect of BACE1 inhibitor on the development of dystrophic axons.

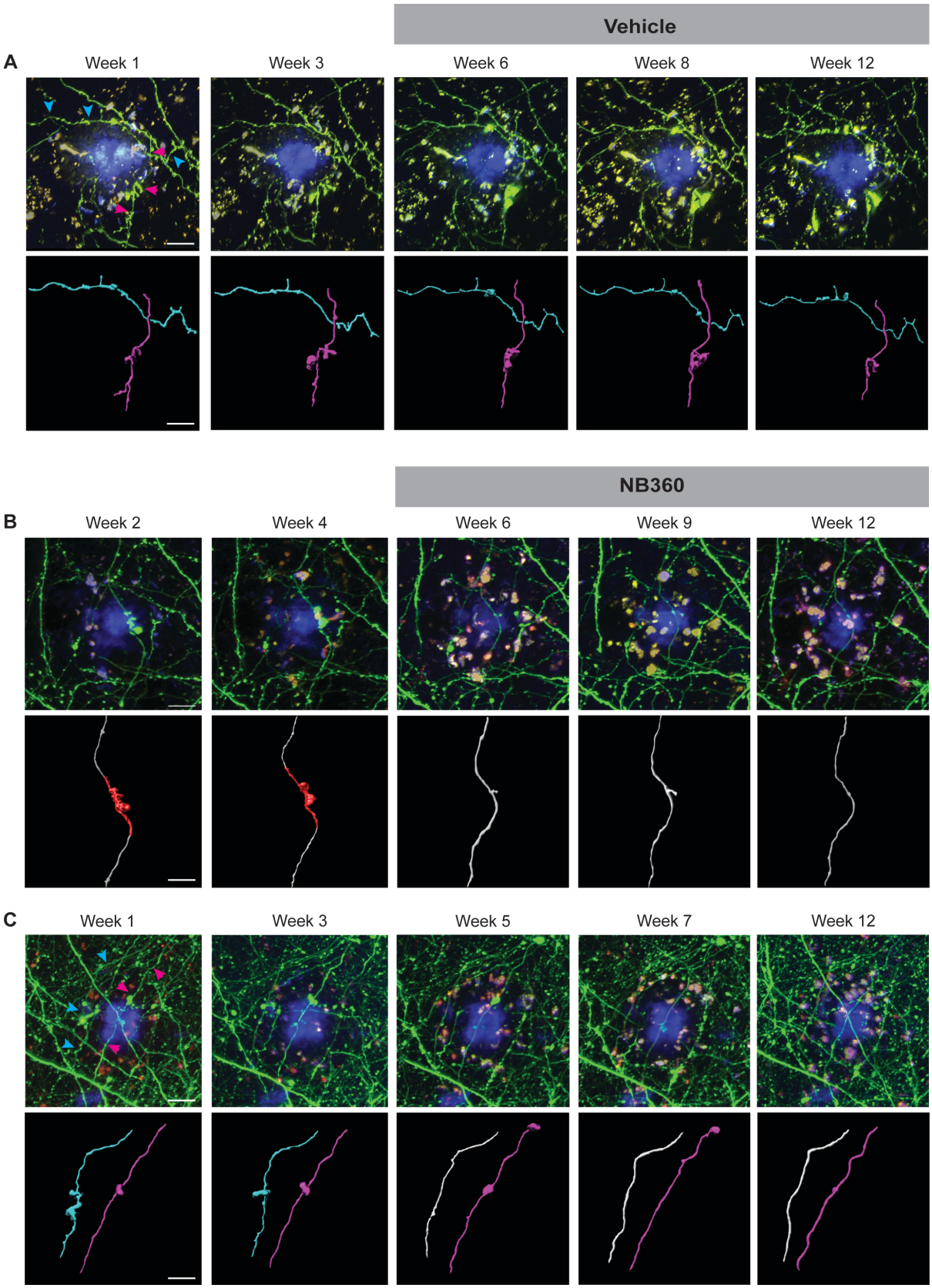


Figure 15: BACE1 inhibitor recovers dystrophic axons. (A-C) Maximum projection of the representative images of GFP-label dystrophic axons taken *in vivo* by 2-photon microscopy before and after NB360 and vehicle treatment. The arrowheads indicate the dystrophic axon and its corresponding 3D reconstruction in the lower panel. Grey color of the axon reconstruction indicates axon recovered (normal axon) by BACE1 inhibition. *Scale bar* 10 µm.

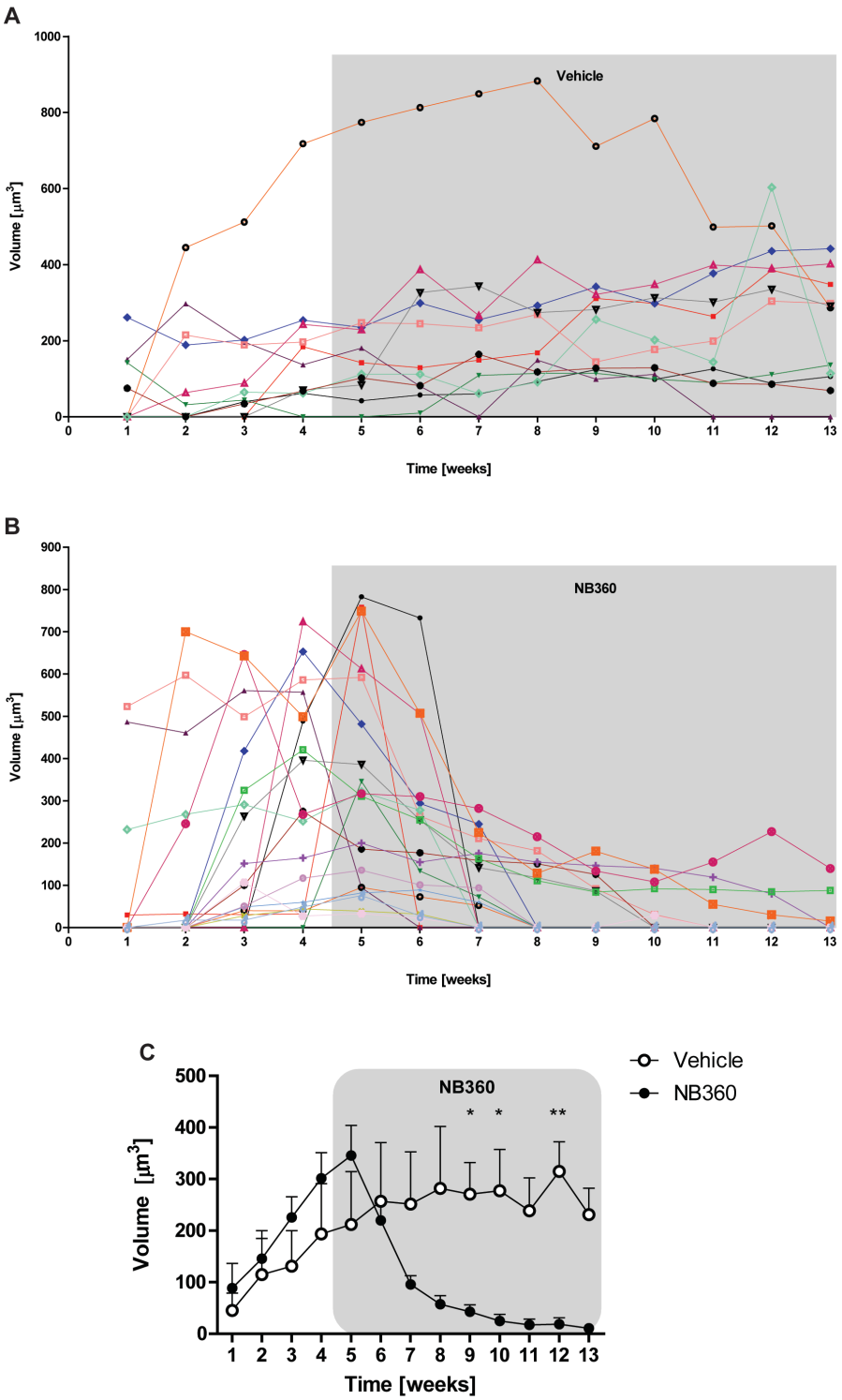


Figure 16: Size dynamics of axonal dystrophies. Changes in the volume of the different axonal dystrophies observed over time during NB360 (A) and vehicle (B) treatment. (C) Mean volume of axonal dystrophies during NB360 and vehicle treatment. Data presented as mean ± SEM, $n = 5$ (NB360 group) and n= 3 (vehicle group); two-way ANOVA with Bonferroni post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4. BACE1 inhibitor affects Aβ plaque growth in cerebral cortex

Aβ plaque formation and growth are major hallmarks of AD pathology and appear to be closely associated with dystrophic neurites and synaptotoxic effects (Fiala 2007; Gouras, Almeida, and Takahashi 2005; Arendt 2005; Scheibel and Tomiyasu 1978). Therapeutic interventions aimed to reduce the production of extracellular Aβ. Based on the constant generation of Aβ from the proteolytic processing of APP mainly by BACE1, the use of BACE1 inhibitors has been emerged as a potential approach for limiting Aβ-mediated presynaptic dysfunctions (Riqiang Yan et al. 2016).

The APPSwe/PSEN1dE9 mice begin to develop Aβ plaques in the cortex by 6 months of age (Jankowsky et al. 2004) which continue growing in a size-dependent manner up to around 12 months old (Monica Garcia-Alloza, Robbins, et al. 2006).

In order to study the presence of Aβ plaques and the associated formation of axonal dystrophies, by long-term two-photon *in vivo* imaging we followed individual plaque growth and formation by measuring the volume over 4 months. Prior to the treatment, we monitored the plaque growth rate, which is considered as the baseline for both NB360 and vehicle-treated mice.

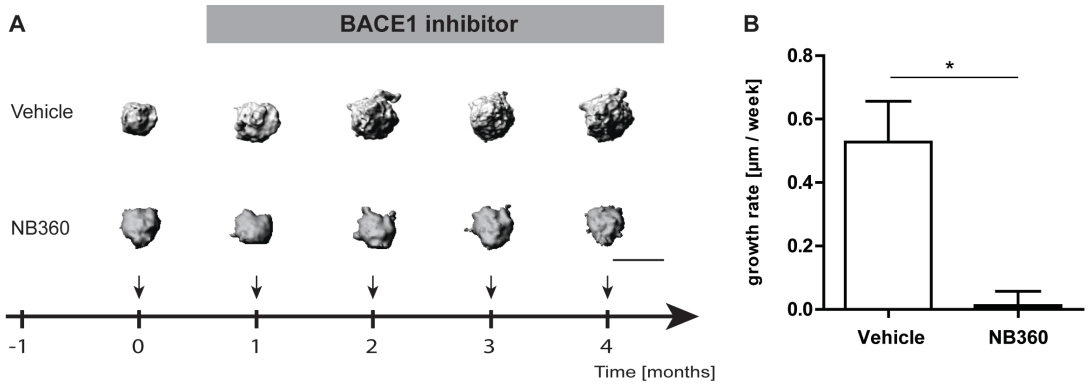


Figure 17: BACE1 inhibitor decreases plaque growth rate. (A) Time series of a single plaque as a surface-rendered object as derived from 3D image analysis. *Scale bar* 20 µm. (B) Comparison of the plaque growth rates resulting from the volume analysis with the values obtained from the vehicle- and NB360-treated mice. Data presented as mean ± SEM, *n* = 5 (NB360 group) and n= 3 (vehicle group); t-test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

The kinetics of amyloid plaque formation and growth were similar to the published studies describing the same or other mouse models (Burgold et al., 2011, 2014; Bittner et al., 2012; Jung et al. 2015) (Figure 17). Aβ plaques can be characterized into large plaques and small plaques. Independently of the deposit size, the NB360-

treated mice showed a remarkably decrease in the plaque growth rate compared with vehicle-treated mice (Figure 17). In addition, mice treated with BACE1 inhibitor exhibited a reduced plaque density compared with vehicle-treated mice (Figure 18) and was not observed the formation of new plaques. Although plaque growth rate was robustly decreased in NB360-treated mice, shrinkage of plaques was not observed. These results agree with complementary findings in the literature (Neumann et al. 2015; Luo and Yan 2010; Devi, Tang, and Ohno 2015; Stamford et al. 2012; Riqiang Yan et al. 2016). Through pharmacological inhibition of BACE1 by NB360, generation of new Aβ peptides is reduced preventing the growth and formation of plaques.

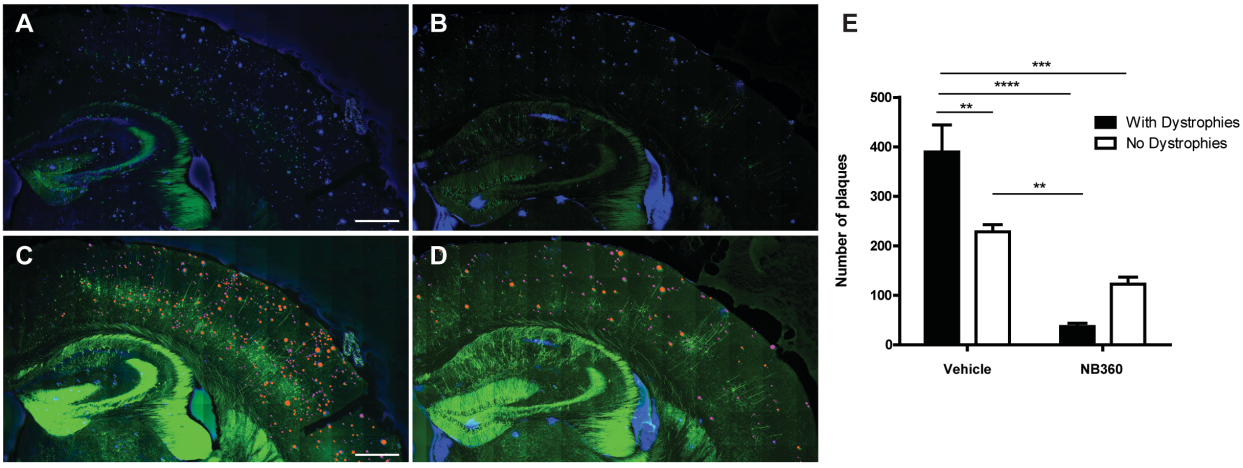


Figure 18: BACE1 inhibition decreases the number of dystrophies associated with amyloid plaques. (A-D) Representative images of eGFP-labeled dystrophic axons in the somatosensory cortex in vicinity to Aβ plaques stained with Methoxy-X04 (blue) (A-B); Orange circles represent the Aβ plaques associated with axonal dystrophies in vehicle- (C) and NB360-treated (D) brain slices. Purple circles represent the Aβ plaques without axonal dystrophies. *Scale bar* 500 µm (E) Comparison between the number of plaques and its association with axonal dystrophies after NB360 treatment. Data presented as mean ± SEM, n = 8 (NB360 group) and n= 6 (vehicle group); two-way ANOVA with Bonferroni post-test. *p < 0.05; **p < 0.01; ***p < 0.001.

5. Inhibition of BACE1 attenuates the progression of axonal pathology depending on the distance between Aβ fibrils and axons

The gradual decrease in the volume of axonal dystrophies during BACE1 inhibitor treatment could be monitored *in vivo* for 3 months. The majority of dystrophic axons analyzed were recovered. As chronic *in vivo* imaging is limited to a certain area of the somatosensory cortex, we investigated the distribution of plaque-associated axonal dystrophies in somatosensory brain slices of both NB360 and vehicle-treated mice. Confocal imaging of axons surrounding amyloid plaques in layer I/II (same region of interest as in the *in vivo* imaging-experiments) was performed in brain slices.

Two remarkable differences between the vehicle- and inhibitor-treated cohorts were found. Firstly, although we detected the presence of axonal dystrophies in NB360-treated mice, the number was notably reduced compared with vehicle-treated mice. Secondly, the number of plaque deposits was significantly reduced in comparison to vehicle-treated mice (Figure 18). Although these observations are consistent with the *in vivo* microscopy data, it did not determine the temporal relationship between reduction of axonal dystrophies and the decreased plaque growth rate during BACE1 inhibition. To resolve this question, we performed high-resolution confocal imaging in brain slices from NB360-treated and vehicle-treated mice that were stained with methoxy-X04 and antibodies against BACE1, Aβ (Aβ4G8) and LAMP1. As expected the pattern of BACE1 immunostaining was similar in both NB360-treated and vehicle-treated brain slices. Our main observation was that Aβ showed dramatic changes in staining intensity and localization in NB360-treated mice. Aβ staining was strongly reduced in NB360-treated mice and localized at the plaque core when compared with vehicle-treated mice (Figure 19). This observation of reduced Aβ staining is consistent with the reduced Aβ levels in both forebrain and plasma after BACE1 inhibition (Figure 12).

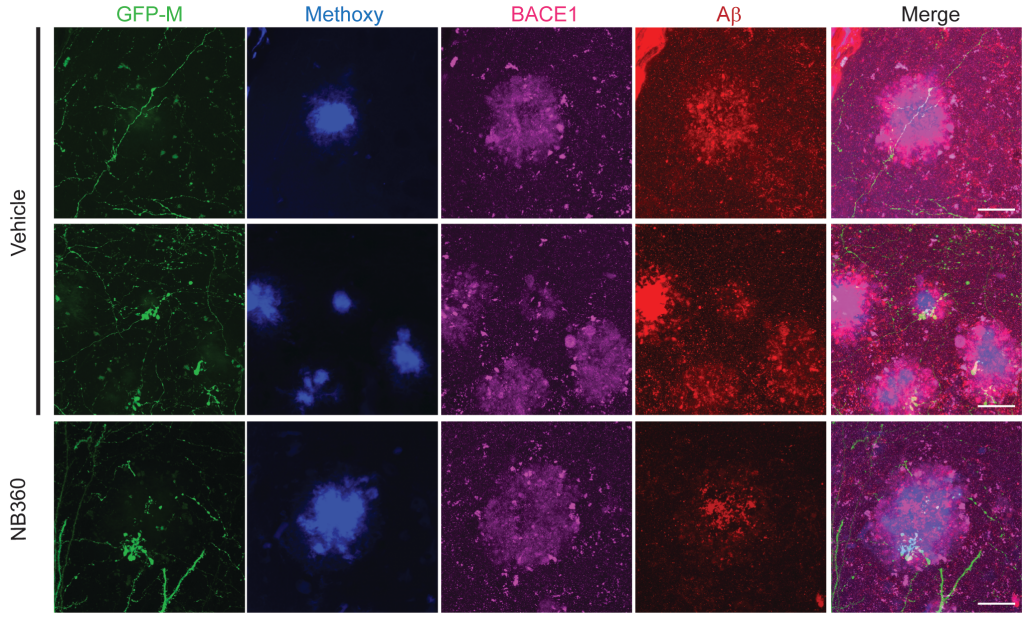


Figure 19: Remaining dystrophies are in contact with Aβ fibrils after BACE1 inhibitor treatment. Immunofluorescence staining with antibodies against BACE1 and Aβ showed that after BACE1 inhibitor treatment, the amount of Aβ is reduced. *Scale bar* 20 µm. All data in this figure were acquired from the cerebral cortices of 10 months old mice.

In addition, given that LAMP1 accumulates at amyloid plaques and reside within the dystrophic axons (Kandalepas et al. 2013; Gowrishankar et al. 2015; Sadleir et al. 2016), we investigate the impact of BACE1 inhibitor on the accumulation of lysosomal membrane proteins at amyloid plaques through a comprehensive immunofluorescent staining analysis of the brain slices of the vehicle and NB360-treated mice. After analysis of the immunofluorescent staining patterns for LAMP1, our major observation was that LAMP1 staining area and localization at amyloid plaques was unchanged by BACE1 inhibition compared to vehicle-treated brains (Figure 20). Although after BACE1 inhibition both plaque growth rate and plaque density are reduced, LAMP1 accumulations are present at all sizes of amyloid plaques (Figure 20). This close relationship between LAMP1 accumulations and amyloid plaques was uniformly observed in both vehicle and NB360-treated brain slices (Figure 20). Visualization of axons via transgenic expression of eGFP showed that axonal dystrophies colocalized with LAMP1 in both vehicle and NB360-treated mice (Figure 20). Likewise LAMP1 accumulations were always found at amyloid plaques where the remaining axonal dystrophies were located. Moreover, axons distant from amyloid plaques were morphologically normal (Figure 20).

In addition, to relate these LAMP1 accumulations to the remaining axonal dystrophies, we examined amyloid plaques with dystrophic axons in NB360-treated

brain slices. We performed 3D reconstructions of axons surrounding plaques and further examined the colocalization of LAMP1 with axonal dystrophies and its distance to amyloid plaques. Interestingly, by analyzing the axons via 3D reconstructions, we observed that the remaining axonal dystrophies are localized in the border and inside plaques (Figure 21) as visualized with Methoxy-X04 staining. Likewise, the remaining axonal dystrophies strongly colocalized with LAMP1 signal while normal axons were not coenriched with LAMP1 (Figure 21G). Thus, although the presence of LAMP1 around plaques has been reported, we demonstrate that normal axons surrounding amyloid plaques, which we consider the recovered axons after BACE1 inhibition, were in close physical association to plaque fibrils (Figure 19 and 20). These observations, in addition to complementary findings in the literature (Kandalepas et al. 2013; Gowrishankar et al. 2015; Sadleir et al. 2016) revealed that pharmacological inhibition of BACE1 is more effective on axonal dystrophies localized at the periphery of the plaque which physical contact with amyloid fibrils is shorter due to the decreased plaque growth.

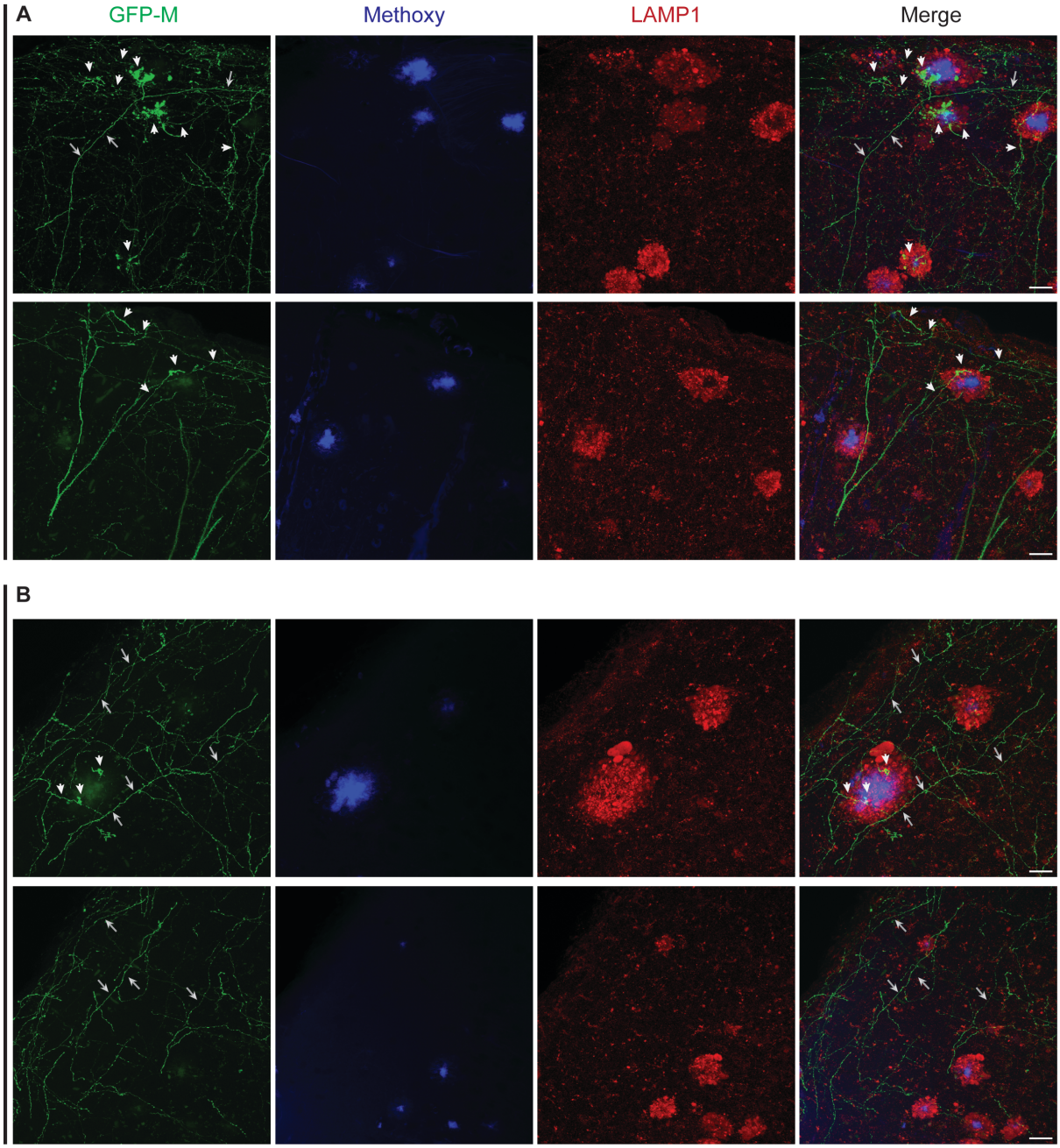


Figure 20: LAMP1 accumulations are present at all sizes of amyloid plaques after BACE1 inhibitor. Representative images of staining for LAMP1 at amyloid plaques (stained with Methoxy-X04) and the surrounding axons in cerebral cortices of vehicle (A) and NB360-treated (B**)** mice. Normal axons are marked by long arrows and dystrophic axons are marked by short arrows. *Scale bar* 20 µm.

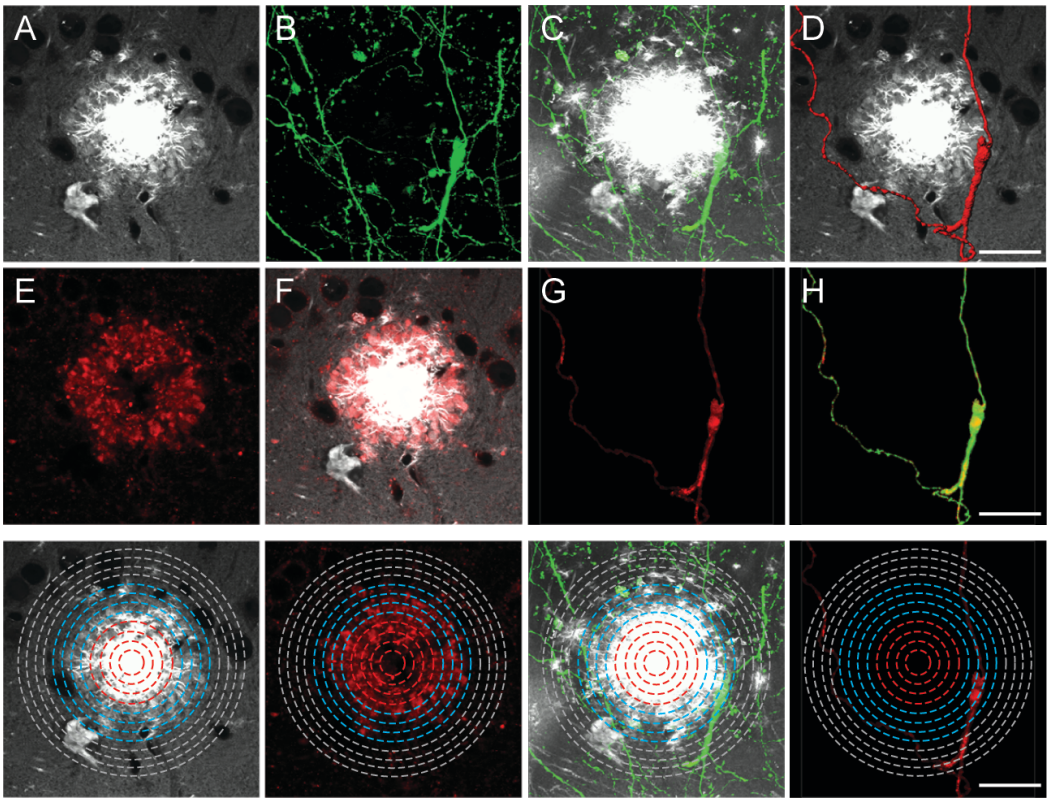


Figure 21: Dystrophies are localized inside and at the border of the plaque. (A) Amyloid plaque stained with Methoxy-X04. (B) eGFP-expressing axons. (C) Superimposed fluorescent image of the plaque and associated axons. (D) 3D reconstruction of a normal axon (left) and a dystrophic axon (right). (E) Immunofluorescence staining for LAMP1. (F) Superimposed fluorescent image of the plaque and LAMP1 staining. (G) LAMP1 intensity in both dystrophic and normal axons. (Lower panel) Distance between the dystrophic axons and its co-localization either with LAMP1 and Aβ plaques. Circles in dashed red line represent the inside of the plaque, circles in dashed blue line represent the border and circles in dashed grey line represent the periphery of the plaque. *Scale bar* 20 µm. All data in this figure were acquired from the cerebral cortices of 10 months old mice.

6. Ultrastructural analysis of the intracellular accumulation of organelles at amyloid plaques

The recovery of dystrophic axons, the reduction in the volume of axonal dystrophies, the decreased plaque growth rate and the robust LAMP1 staining within axonal dystrophies after BACE1 inhibition, suggested that BACE1 inhibitor is less effective on dystrophic axons which dystrophies are localized inside of the plaque. To investigate this possibility at a structural level, we examined sections of vehicle and NB360-treated mouse brain by electron microscopy (EM). We found dystrophies contacting amyloid plaques in both vehicle (Figure 22A) and NB360-treated (Figure 22B) sections. Accordingly, upon a higher magnification, EM revealed the presence of a diversity of putative autophagic vesicles (AVs) within dystrophies that immediately surround amyloid plaques in both vehicle (Figure 22C-E) and NB360-

treated (Figure 22F-H) sections. These AVs have distinct morphologies such as heterogeneous electron dense double-membrane vesicles, electron dense compacted amorphous vesicles, multilamellar vesicles, translucent vesicles and amorphous electron dense vesicles. The most common morphology of AVs present in dystrophies is electron dense compact amorphous and multilamellar vesicles. Overall, we found a substantial accumulation of AVs within dystrophies surrounding amyloid plaque, in both vehicle and NB360-treated sections, indicating that BACE1 inhibition does not alter the intracellular accumulation of organelles within dystrophies that contact the amyloid plaque.

Given that the physical contact of axons with amyloid plaques causes the formation of dystrophies, our results suggest that during BACE1 inhibition, the concentration of toxic Aβ species and aggregation into insoluble β-sheet amyloid fibrils is diminished, decreasing plaque growth. Thus, physical contact between amyloid fibrils and both dystrophic and normal axons is reduced. Therefore, the accumulation of BACE1, APP and autophagic vesicles as well as axonal swellings decreases. Microtubules can support the normal axonal transport of BACE1, vesicles and other organelles leading to the recovery of the dystrophic axons and prevention of dystrophies formation (Figure 23).

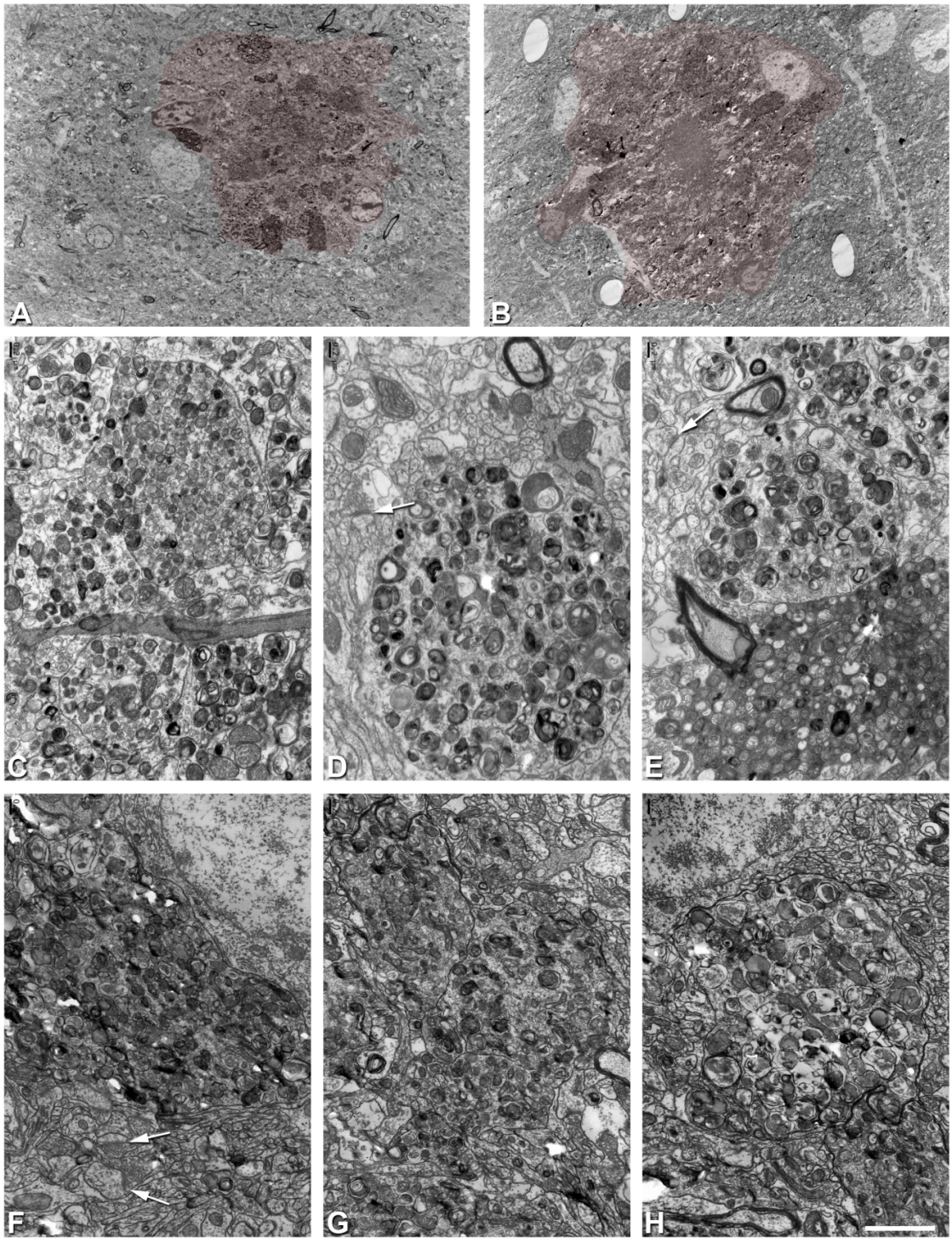


Figure 22: Ultrastructural analysis of organelle accumulation surrounding amyloid plaques. (A) Electron panoramic micrograph of vehicle-treated cerebral cortex tissue showing a central amyloid plaque (marked in red) surrounded by dystrophies. (B) Electron panoramic micrograph of NB360-treated cerebral cortex tissue showing a central amyloid plaque (marked in red) surrounded by dystrophies. (C-E) Higher magnification of the dystrophies from plaque in A reveals the morphology of organelles that accumulate within cellular processes that contact the amyloid plaque. (F-H) Higher magnification of the dystrophies from plaque in B reveals the morphology of organelles that accumulate within cellular processes that contact the amyloid plaque. Arrows point out synaptic contacts. *Scale bar* 8 µm.

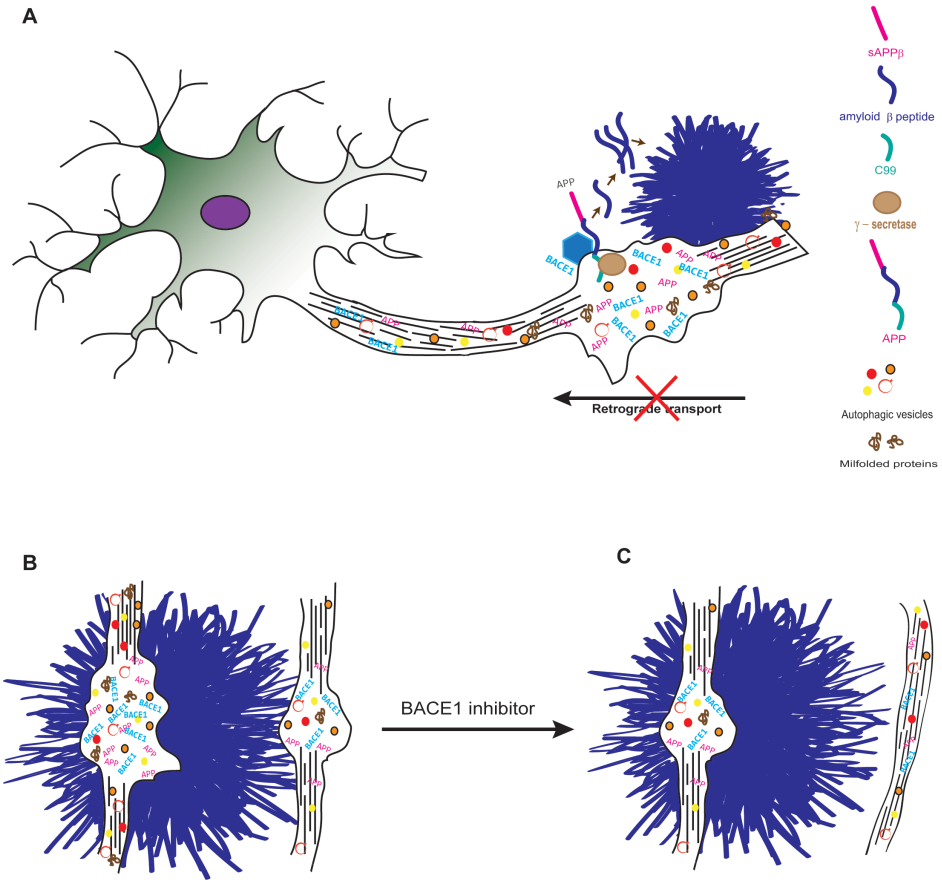


Figure 23: Working hypothesis. (A) Schematic diagram that summarizes the formation of the axonal dystrophies in AD and (B-C) how BACE1 inhibition can recover dystrophic axons associated with plaques. (B) Dystrophic axons are localized inside, at the border or at the periphery of the amyloid plaque. (C) After BACE1 inhibition, the physical contact between amyloid fibrils and axons localized at the periphery is reduced facilitating the recovery of the dystrophic axon.

DISCUSSION

BACE1 levels and activity are increased in postmortem brains and CSF from AD brains (Fukumoto et al. 2002; Holsinger et al. 2002; Tyler et al. 2002; Yang et al. 2003; Li et al. 2004; Harada et al. 2006). Accumulation of BACE1 begins to increase in parallel with amyloid pathology and is observed predominantly in dystrophic presynaptic terminals surrounding amyloid plaques in brains of AD patients and AD mouse models (Zhao et al. 2007; Kandalepas et al. 2013). The amyloid cascade hypothesis of AD postulates that the overproduction, deposition and/or reduced clearance of Aβ peptides in the brain is a key event that triggers the synaptic degeneration and ultimately the deterioration of cognitive function and memory (J. Hardy and Selkoe 2002; Lacor et al. 2007). Given that BACE1 is the required therefore rate-limiting enzyme for Aβ generation and (R. Vassar et al. 1999; Ghosh, Brindisi, and Tang 2012), it represents one of the prime targets for the development of disease-modifying drugs in AD. Several BACE1 inhibitors are currently in advanced phases of clinical trials (Lucas, Fukushima, and Nozaki 2012; May et al. 2011). However, the development of drugs with a favorable safety profile that pharmacologically inhibit BACE1 has a long way to go before they are applicable in the treatment of AD (Yuan et al. 2013; Riqiang Yan and Vassar 2014; Riqiang Yan et al. 2016; Robert Vassar and Kandalepas 2011).

In recent years there has been considerable debates regarding how plaques act as a neurotoxic agent in AD, and how BACE1 elevation is triggered by amyloid pathology. Although much data exist on the accumulation of BACE1 in swollen dystrophic axons surrounding plaques and its association with lysosomal dysfunction (Vickers et al. 1996; Zhao et al. 2007; Zhang et al. 2009; Kandalepas et al. 2013), two recent comprehensive studies provided new insights into the fundamental mechanisms of presynaptic dystrophy formation driven by extracellular Aβ deposits. Importantly, they revealed that the formation of axonal dystrophies is caused by a feed-forward mechanism of increased accumulation of BACE1, APP and lysosomes (preferentially lysosome precursors). This is a result of a local impairment in the retrograde axonal transport triggered by Aβ generation and plaque progression which mediate axonal microtubule disruption and microtubule-based transport impairment (Gowrishankar et al. 2015; Sadleir et al. 2016).

Previous studies have reported treatment strategies for the modulation of plaque

associated-axonal dystrophies (M. Garcia-Alloza et al. 2007; R. P. Brendza et al. 2005; T. L. Spires 2005; Tsai et al. 2004; D'Amore et al. 2003). However, these studies did not monitor the formation of axonal dystrophies and plaque growth kinetics over an observation period of 4 months including a long-term treatment.
To investigate the pharmacological effect of BACE1 inhibition on the dystrophic neurites formation and plaque progression, we combined chronic *in vivo* two-photon imaging and confocal imaging in mice treated with BACE1 inhibitor. Fortunately, transgenic mouse models recapitulate some of the pathological aspects of AD allowing the analysis of disease initiation and progression. In our study, we observed that around 21% of the axons near plaques developed dystrophies. The 3D reconstructions of the eGFP-expressing dystrophic axons enabled us to observe that dystrophies have different sizes and morphology. Moreover, as shown in vehicle-treated mice, dystrophies do not grow regularly but they can increase and decrease their volume over time (Figure 16A). Furthermore, we show that chronic treatment with the novel BACE1 inhibitor, NB360, rescues the majority of plaque-associated axonal dystrophies in the brains of APPSwe/PSEN1dE9 x GFP-M mouse models (Figure 16B).

Two important conclusions emerge from our study:

1) Reduction of axonal BACE1 activity attenuates the local generation of Aβ in the vicinity of the amyloid deposits and thus facilitates the recovery of axonal dystrophies and prevents formation of new dystrophies.
2) Decreasing plaque growth rate prevents the toxic effects derived from Aβ generation in the neighboring axons and the formation of new plaques.

Taken together, as illustrated in Figure 23, our results suggest the following hypothesis regarding the effect of BACE1 inhibition on axonal dystrophies formation and plaque progression. Treatment with BACE1 inhibitor reduces Aβ generation and plaque growth. This effect results in a reduced physical contact with the surrounding normal and dystrophic axons. Therefore, the decreased neurotoxicity stems from a reduction of either soluble Aβ oligomers or insoluble Aβ fibrils, benefitting the stabilization of microtubules and promoting the extension of newly polymerized microtubules from the distal part of the dystrophy towards the region close to the

plaque. Microtubule-based axonal transport is re-established and vesicular cargoes proximal and distal to the plaque are transported. Because BACE1 is normally degraded by lysosomes, (Koh et al. 2005; Ye and Cai 2014; Kandalepas et al. 2013) its transport to lysosomes for degradation is efficient and vesicles containing BACE1 and APP accumulate less at the axons after treatment. After treatment, BACE1 levels and turnover through retrograde transport are regulated controlling APP processing and Aβ generation in the dystrophic region of the axon. Axonal swellings decrease gradually thereby reducing physical contact with Aβ fibrils preventing the formation of new axonal dystrophies. Our hypothesis is supported by previous studies that detected a partial reduction of the dystrophic neurites surrounding plaques using immunotherapy (Lombardo et al. 2003) or antioxidant treatments (Monica Garcia-Alloza, Dodwell, et al. 2006; M. Garcia-Alloza et al. 2007).
Thus, BACE1 inhibition is beneficial in slowing down the progression of dystrophic pathology associated with plaque growth, improving functional synaptic transmission and cognitive functions.

Why are some dystrophies still present after long treatment with BACE1 inhibitor?

The spherical and extracellular Aβ plaques are a pathological feature of AD. After their formation, they can be morphologically characterized as dense-core plaques and diffuse plaques (Fiala 2007).
Despite the extensive overproduction and deposition of Aβ as the sources of neurotoxicity in AD mouse models and in AD, ongoing debates about the steps of the amyloid cascade hypothesis have been suggesting mature fibrils as the cause of disease pathogenesis. This hypothesis is based on the findings that amyloid plaques are associated with neuritic dystrophies and synapse loss (D'Amore et al. 2003; Monica Garcia-Alloza, Dodwell, et al. 2006; Lombardo et al. 2003; Urbanc et al. 2002; Lorenzo and Yankner 1996; Stéphan, Laroche, and Davis 2001; Cohen et al. 2013).
Dense-core plaques are defined as fibrillary amyloid deposits with a compact core and associated with axonal dystrophies embedded within the dense-core or in the vicinity of the plaque (Masliah et al. 1994; Su, Cummings, and Cotman 1998; T. C. Dickson and Vickers 2001; Shah et al. 2010). The microenvironment within and

around these dense-core plaques is considered toxic compared with diffuse plaques, and it is favorable for additional plaque development contributing to the amyloid cascade pathology (Selkoe 1991; Urbanc et al. 2002; J. Hardy and Selkoe 2002; Bero et al. 2011; Beker et al. 2012; Alberto Serrano-Pozo et al. 2016).

Although dense-core plaques are defined by the presence of insoluble Aβ fibrils, they also display a peripheral halo composed by soluble and toxic oligomeric intermediates (Shankar et al. 2008; Koffie et al. 2009; Hong et al. 2011; Koffie et al. 2012).

Therefore, preventing the production and limiting the aggregation of Aβ through inhibition of BACE1 may prevent or limit the progression of the pathology associated with plaque growth.

In the present work we were able to monitor the effect of chronic BACE1 inhibition on plaque density and plaque growth kinetics over time in a mouse model of AD. In agreement with (Neumann et al. 2015), we observed that inhibition of BACE1 by NB360 reduced the generation of Aβ peptides (Figure 12) and plaque growth rate (Figure 17). However, we did not observe a reduction of the size of the existing plaques. These data support the idea that BACE1 inhibition limits the production of new Aβ and therefore reduces the density of new-formed plaques, while the effect on plaque regression is limited.

As already mentioned, plaques are considered to be responsible for the abnormal swellings of the surrounding neurites (Knowles et al. 1999; D'Amore et al. 2003; Monica Garcia-Alloza, Dodwell, et al. 2006). Considering this, we extended our *in vivo* observations and assessed the effect of BACE1 inhibition on axonal dystrophies *ex vivo* using high-resolution confocal imaging. This approach allowed us to explore, in more detail, plaques and surrounding axons, which were not possible to observe due the limited area of the *in vivo* imaging. In agreement with our *in vivo* imaging experiments, we observed the presence of axonal dystrophies after the long-term treatment with BACE1 inhibitor (Figure 18 and 19). Moreover, these remaining dystrophies were located within the dense-core of the plaque and co-localized extensively with LAMP1 (Figure 20 and 21).

Previous EM analysis indicated that the axonal dystrophies are heterogeneous containing electron dense and multilamellar vesicles that may be autophagic/lysosomal intermediates (Kandalepas et al. 2013; Gowrishankar et al. 2015). Although our observation at amyloid plaques from vehicle and NB360-treated

sections did not show visual differences in the content of organelles within dystrophies (Figure 22C-H), it supports our hypothesis regarding the reduced effect of BACE1 inhibitor on dystrophies that contact amyloid plaques. Further studies are needed to better characterize the heterogeneity of dystrophies after BACE1 inhibition, and determine its causes and consequences. Besides, further studies in order to characterize the type of axon and its susceptibility to recover after BACE1 inhibitor will further elucidate the mechanisms whereby BACE1 inhibition recovers dystrophic axons.

Taken together, our *in vivo* and *ex vivo* results suggest that pharmacological inhibition of BACE1 is more effective in the periphery the plaques through reduction of the Aβ generation and fibrillization, thus diminishing the propensity to form dystrophies. However, has less effect on axonal dystrophies located within the dense-core of the plaques, even with reduced Aβ production. This demonstrated that the microenvironment within the core of the plaques remains toxic becoming more difficult to recover the existing pathology there.

Our data suggest that pharmacological inhibition of BACE1 limits the generation of Aβ and thus reduces plaque growth. Accordingly, diminishing the formation of neurotoxic fibrils prevents the progression of presynaptic dystrophic neurites surrounding plaques.

BACE1 inhibition in mouse models of AD – medical relevance

BACE1 inhibitors are designed to target one of the key drivers of AD progression. Although this study was conducted in mice, it shows promise for AD treatment. Abnormal accumulation of both BACE1 and APP in dystrophic axons that surround amyloid plaques (Zhao et al. 2007; Zhang et al. 2009; Kandalepas et al. 2013), suggest that peri-plaque Aβ production accelerate amyloid deposition and induce a vicious pathogenic cycle (Torres et al. 2012; Sadleir et al. 2016). Therefore, decreasing BACE1 activity in peri-plaque dystrophic axons represents a potentially efficacious therapeutic goal of BACE1 inhibition. As shown by the present study, BACE1 inhibition in an AD mouse model decreased the Aβ production and significantly reduced the plaque growth. As a result, the formation of new amyloid plaques was prevented and plaque-associated dystrophic axons have recovered.

Despite the challenges of BACE1 inhibitor drug development over the last years, in particular the level of BACE1 inhibition, the introduction of several BACE1 inhibitors into clinical trials promise a therapeutic approach for AD. Currently, some BACE1 inhibitors are in phases 1, 2 and 2/3 (Riqiang Yan and Vassar 2014), although two BACE1 inhibitors have been terminated due to their toxicity.

However, important questions concerning therapeutic goals and outcomes remain to be answered for future clinical development of BACE1 inhibitors for AD. Given that Aβ deposition starts years before the clinical diagnosis of AD and cause the formation of axonal dystrophies surrounding amyloid plaques, it raises the questions at what stage of AD should be administered the BACE1 inhibitor and how is the optimal efficacy of treatment. At this time, this study using the new BACE1 inhibitor (NB360) added new insights into the relationship between BACE1 inhibition, Aβ reduction, plaque load and axonal pathology associated with plaques, in order to estimate the levels of BACE1 inhibition need at a given stage of AD for clinical development.

In this study is shown for the first time that treatment with a BACE1 inhibitor reduced the axonal pathology associated with plaques. The NB360 (Neumann et al. 2015) used in this study, is not yet developed for clinical trial, but taking into account the results of our study raise hopes for future clinical development of BACE1 inhibitors for AD.

Although BACE1 inhibitors still have a long way to go before they are applicable in the battle against AD, they can at least be used to prevent and slow the progression of the disease.

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