UNIVERSITY OF CALIFORNIA SAN DIEGO

The GSM BPN-15606 as a Potential Candidate for Preventative Therapy in AD

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Olga Prikhodko is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this dissertation to my family; I knew some of you when this began, and the rest of you I found along the way.

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

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by

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Alzheimer's disease (AD) is a degenerative brain disease, and the most common form of dementia in the elderly, which affects about 5.5 million individuals in the US in 2017. This number will only continue to grow as the proportion of population aged 65 years or older increases due to extending life span resulting from better medical care; currently, 10% of people in this group have AD. AD is the sixth leading cause of death in

the US; deaths from this disease increased 89% from 2000 to 2014. The national cost of the disease was estimated to be $230 billion in 2017, which included significant unpaid time away from work for assisted care from friends and family of the patient, costs for providing long-term health care and for currently available palliative treatments which are limited to temporary and mild alleviation of cognitive and behavioral symptoms in a subset of patients. There is no cure, effective prevention or therapeutic regimens for altering the course of the underlying disease process.

Chapter 1 of this dissertation summarizes the current understanding of Alzheimer's disease, the molecular pathways behind its incidence, available symptomatic treatments, and therapeutic options that are in clinical trials.

Chapter 2 addresses the aim of this dissertation, which was to test whether a γ-secretase modulator, BPN15606, is an effective disease-modifying or preventative treatment in the PSAPP mouse model of AD. We have found that BPN-15606 prevented cognitive impairment, reduced amyloid plaque load, microgliosis and astrogliosis associated with the AD phenotype of PSAPP mice when administered to pre-plaque, but was ineffective when administered to post-plaque PSAPP mice No treatment-related toxicity was observed. We have thus concluded that BPN-15606 is a viable preventative therapeutic for AD, and is a candidate for early-phase human safety trials.

Chapter 3 provides recommendations for future studies to further define the mechanism of γ-secretase modulation by BPN-15606. Additionally, it addresses the need for development and validation of combination therapies to address the multifaceted pathology of AD, and of reliable biomarker techniques to diagnose preclinical AD.

CHAPTER 1

Introduction

1.1 Alzheimer's disease

Alzheimer's disease is a neurodegenerative disease that is characterized by progressive memory loss, language and planning problems, and difficulty performing basic tasks[1]. It is the sixth leading cause of death in the United States [2]. As of 2019, 5.8 million people are living with AD in the US, and this number is expected to continue to rise as life expectancy increases and the baby boomer generation reaches 65 years of age, when the incidence of AD rises [3]. An estimated 18.6 billion hours of informal care is provided by caregivers for AD and other dementia patients in 2018; this care is estimated to have a value of $234 billion, as 83% of these caregivers are unpaid family members and friends [4]. Beyond a monetary burden, this invaluable service also imposes negative mental and physical consequences on the care providers, such as stress, depression, susceptibility to diseases and other health complications [5, 6]. While there are currently no cure or disease-modifying treatments, future interventions that slow or reduce the symptomatic features of AD will significantly reduce national costs and the burdens to caregivers [7, 8].

Most incidences of AD are sporadic, with less than 1% accounted for with familial mutations (in APP, amyloid precursor protein, or PSEN1/PSEN2, presenilin) or gene variants (the e4 allele of APOE, apolipoprotein E) [9, 10]. There are many risk factors to developing AD, such as poor cardiovascular health, smoking, diabetes, lack of mental stimulation, and traumatic brain injury; however, the primary risk factor for AD is

age [11-16]. 16% of people of 65 to 74 years of age have AD, and this percentage increases in older age brackets [9].

One of the issues surrounding finding treatments for AD is that pathological changes in the brain precede symptom onset by 20 years. These changes start with amyloid β plaque deposition, followed by tau-mediated neuronal injury and dysfunction and finally the brain structure alters [17-19]. AD is characterized by cholinergic basal forebrain (CBF) neuron loss and reduced cortical choline acetyltransferase (ChAT) and acetylcholinesterase activity (AChE), which leads to cognitive decline [20]. Minimizing the effect of loss of these systems is the basis behind current FDA-approved AD medications [21]. The available symptomatic therapies are cholinesterase inhibitors (rivastigmine, galantamine, donepezil) and the *N*-methyl-D-aspartate receptor antagonist memantine [3]. The cholinesterase inhibitors are beneficial to patients with mild-to-moderate AD [22-24], while memantine is the only drug that has been approved for moderate to severe AD [25]. The combination of memantine and donezepil is also used for moderate to severe AD with better clinical outcomes [26]. These drugs are generally well tolerated and provide temporary symptomatic relief, but none target the cause of the disease.

The amyloid cascade hypothesis is at the core of AD; it states that the imbalance between Aβ42 production and clearance leads to its oligomerization and deposition into plaques, leading to hyperphosphorylation of tau, synaptic loss, persistent neuroinflammation, and neuronal injury [27, 28]. The first genetic mutations that caused AD were discovered in the APP gene [29, 30]. These disease-causing mutations are

clustered near the sites of α-, β- or γ-secretase cleavage, often promoting processing through the amyloidogenic pathway, discussed in a later section [31-33]. Other APP mutations that have been associated with AD are within the Aβ motif of APP, and heighten the ability of the Aβ to aggregate [33]. Disease-associated mutations in APP-processing enzymes also tend to shift APP processing towards the amyloidogenic pathway [34]. Some of the criticisms of this hypothesis include the observation that the level of plaque load in a patient's brain does not directly correlate with the degree of cognitive impairment, and some individuals have cortical Aβ deposits, but do not develop dementia. However, further characterization of the types of Aβ (soluble oligomers vs. insoluble fibrils) and the ratio of Aβ42 to Aβ40 refined the link between forms of Aβ and disease severity [35-38]. Another criticism of the hypothesis is that AD mouse models with the APP transgene do not exhibit neuronal loss or neurofibrillary degeneration [39]; however, a double mutant tau/APP mouse model develops enhanced neurofibrillary degeneration compared to tau-only model, supporting the link between Aβ accumulation and tau pathology [40]. Also, our current AD mouse models are far from perfect; the time scale of mouse model AD pathology is vastly different from AD occurring in humans, and the immune response to AD pathology in mice uses the mouse, rather than the human, inflammatory mediators that greatly contribute to disease features [41].

1.2 APP Processing

APP (amyloid precursor protein) is a type I integral membrane protein, whose gene maps to chromosome 21. It has three major isoforms derived from alternative splicing (APP751, APP770, and APP 695, the latter being highly expressed in the brain

[42-44]). It is also expressed in the thymus, heart, muscle, kidney, liver, spleen, skin, lung, intestine, and adipose tissues.

APP is made in the endoplasmic reticulum and transported through the Golgi apparatus to the trans-Golgi-network, where most APP is located [45]. From there, it can be transported to the cell surface, where it's cleaved by α-secretase, or re-internalized by the endosomal\lysosomal degradation pathway [46].

Amyloid precursor protein processing involves two competing pathways: the non-amyloidogenic, involving sequential cleavage of APP by α- and γ-secretases, and the amyloidogenic, where APP is cleaved by BACE1 (β-site APP cleaving enzyme 1), then γ-secretase. These processes are carried out by neurons and astrocytes in the brain (microglia lack a meaningful expression of BACE1, though they express γ-secretase). Some non-neural tissues, such as intestinal epithelium, skin, and skeletal muscle, also secrete Aβ [47].

1.2.1 Non-amyloidogenic pathway

The first enzyme of the non-amyloidogenic pathway is α-secretase, a metalloproteinase, and it cleaves APP at the plasma membrane to form an N-terminal cytoplasmic neuroprotective sAPP-α fragment and the C-terminal C83 (Fig. 1.1). The latter is then cleaved by γ-secretase to form AICD (APP intracellular domain) and the extracellular p3 fragment (17-40/42) [48].

1.2.2 Amyloidogenic pathway

In the amyloidogenic pathway, BACE1 cleaves APP to release the N-terminal sAPP-β cytoplasmic fragment and C99, which stays in the plasma membrane (Fig. 1.1).

BACE1 is present at the Golgi membrane and in endosomes, requiring an acidic environment for optimal activity, but can also be found at the cell surface. It is the rate-limiting step in the amyloidogenic pathway. The C99 is then cleaved by γ-secretase in sequential cleavages (epsilon, zeta, and gamma). Depending on the position of the epsilon cleavage of C99, an AICD (APP Intracellular Domain) along with either Aβ49 or Aβ48 are generated. The latter are further processed into two major product lineages, Aβ49→ Aβ46→ Aβ43→ Aβ40→ Aβ37 or Aβ48→ Aβ45→ Aβ42→ Aβ38 [49, 50]. In the cerebrospinal fluid (CSF), Aβ 1-40 is the most abundant (at 2-3 ng/mL) and Aβ 1-42 is the second most abundant (0.75 ng/mL) [51, 52]. Amyloid β fragments are then secreted into the extracellular space and cleared by CSF and vascular system; in mice, this turnover has a half-life of 0.7-2 h [53, 54]. Elevated Aβ42/Aβ40 ratio is associated with AD pathology, and in FAD, this is due to either an increase in Aβ42 or a decrease in Aβ40 levels [55]. Mutations in APP contribute to destabilization of γ-secretase/APP-C99 complex contributing to the release of longer Aβ peptides [56]. Soluble Aβ fragments self-assemble into soluble oligomers, then insoluble fibrils, which then can precipitate into insoluble Aβ plaques, resistant to proteolysis. Oligomers and plaques have shown to be toxic to brain cells [57].

1.3 γ-secretase structure and function

γ-secretase has more than 200 mutations associated with familial AD [58]. γ-secretase is a multimeric aspartyl protease composed of four subunits: presenilin (PS1 or PS2), nicastrin (NCT), presenilin enhancer 2 (PEN2) and anterior pharynx defective 1 (APH1). *PSEN*1 or 2 are the genes encoding the catalytic subunit of the complex, NCT

helps maturation of the complex, Aph-1 stabilizes the PS1-NCT complex, and PEN2 is the last component to bind [59-61]. The endoproteolysis of PS1 into PS1-NTF and PS1-CTF requires PEN2 and is necessary for the catalytic activity of the complex [62, 63]. The PS1delta exon 9 mutant is constitutively active without the requirement of endoproteolysis; this mutation is present in the PS1/APP AD mouse model [64].

γ-secretase activity is localized to the plasma membrane and endosomes [65]. It has about 90 known targets, which are type 1 membrane proteins [66]. These targets are involved in cell fate determination, cell adhesion and migration, synapse formation, axon guidance, and neurite outgrowth [66]. γ-secretase makes sequential cleavages, starting with the endoproteolytic epsilon cleavage, releasing a soluble intracellular domain, and continuing with exopeptidase-like gamma-cleavages until the substrate is released [67]. The processivity of the enzyme is determined by various conditions that affect enzyme-substrate (E-S) stability, such as mutations [56] and modulators [68]. Greater stability of the E-S complex leads to shorter end products; in the case of Aβ peptides, the shorter products are less prone to aggregation and thus are more benign. FAD-linked presenilin/γ-secretase mutations affect γ-secretase processivity by destabilizing the E-S complex, which leads to the release of longer Aβ peptides [69-71].

1.4 Role of Aβ

The imbalance between Aβ production and clearance is at the crux of Alzheimer's disease, leading to downstream tau tangles, persistent neuroinflammation, neuronal loss and cognitive decline. However, as with most pathways in an organism, Aβ peptide production has a role in maintaining homeostasis in non-disease conditions. 60-70% of

all vertebrates produce Aβ [72] and the sequence of Aβ is 95% conserved among mammals [73]. Additionally, depletion of endogenous Aβ in animal models led to impaired short- and long-term memory retention [74-76], reduced hippocampal LTP and PTP [75, 76] and microhemorrhages [77]. Recent evidence has brought to light antimicrobial and antiviral functions of Aβ, as well as its role in regulating synaptic function.

1.4.1 Immune system

The idea that Aβ is an antimicrobial peptide (AMP), and Aβ deposition is a protective innate immune response, is supported by several studies [78-82]. Amyloid fibril generation disrupts microbial cell membranes [83], neutralizes bacterial endotoxins [84] and entraps pathogens [85-87]. Fibrillization in response to an infection can thus accelerate β-amyloid deposition and is especially dangerous in a person who has an imbalance of Aβ production and clearance due to mutations in AD-related genes or other factors.

1.4.2 Cell adhesion

In the mammalian epidermis, APP is predominantly expressed in basal keratinocytes, with moderate expression in melanotyes and melanoma cells [88, 89]. In these cell types, evidence points to APP's role in cell adhesion via its interaction with perlecan, laminin, collagen type IV, and entactin.

1.4.3 Synaptic function

Depletion of endogenous Aβ in rats reduces LTP and short- and long-term memory, which is rescued by human Aβ42 supplementation [74]. However, the duration

of exposure to Aβ42 is important, as short-term treatment enhances neuronal plasticity, while longer-term treatment reduces it *in vitro* – a finding that was reflected in *in vivo* studies in terms of contextual memory [90]. Low (picomolar) Aβ concentrations directly activate α7-nicotinic acetylcholine receptors, enhance LTP and memory consolidation, while higher (nanomolar) Aβ concentrations inhibit the receptors and impair memory; this effect is absent if the α7-nicotinic acetylcholine receptors are inhibited prior to treatment [91-93]. Aβ also triggers the elimination of excitatory synaptic connections at nanomolar doses, and impairment of NMDAR Ca^{2+} entry at picomolar doses [94]. The mechanism of this is yet unknown, but Aβ interacts with a number of membrane receptors besides α7 nicotinic acetylcholine receptors [95] and NMDARs [96], such as AMPARs [97], LilrB2 [98] and amylin receptor [99].

1.5 Anti-amyloid therapies

Active immunizations for the treatment of AD employ the administration of an AD-specific antigen, like fibrillar Aβ42, with an adjuvant, which induces the organism's own immune system to develop an antibody response. Preventative administration of Aβ42 protected AD mouse models from developing disease-associated memory deficits [100-102]. The first clinical trial of an AD vaccine, AN-1792, which used fibrillar Aβ42, demonstrated that the subjects with high anti-Aβ antibody titers also had reductions in AD brain pathology after autopsy, though there was no reduction of soluble oligomeric forms of Aβ, nor evidence of improved survival or time to severe dementia [103-105]. The trial was halted after a portion of patients receiving the vaccine developed aseptic meningoencephalitis associated with autoreactive T-cell infiltration into the brains of

immunized subjects [103, 106]. Only a quarter of patients responded to the vaccine, and the anti-Aβ antibody titers were overall low; this can be explained with reduced immune system response to a self-antigen, and low responsiveness of older people to vaccines [107, 108]. Thus, an effective active immunotherapy has to be administered before the accumulation of toxic forms of Aβ, the patients' immune system has to adequately respond to the vaccine, and the vaccine cannot recruit autoreactive T-cells to the brain. Currently, active immunotherapies in trials are CAD106 [109, 110], UB-311 (NCT03531710), AD03 (NCT01401582), and LUAF20513 (NCT02388152).

Passive immunotherapies for amyloid β involve the administration of exogenous antibodies; consistent titers can be achieved, though by repeated administration, bearing associated production costs [111]. A few monoclonal antibodies have advanced to clinical trials, but failed for various reasons, which give us further insight into the physiologic function of Aβ.

Bapineuzumab is a humanized version of murine IgG1 anti-Aβ monoclonal antibody (mAb), which bound both soluble and fibrillar Aβ and induced microglial phagocytosis of Aβ [112]. In phase 2 trial, 17% of participants developed ARIA-E, and 47% of affected with ARIA-E (amyloid-related imaging abrormalities – edema) also developed ARIA-H (hemorrhage). This incidence was significantly related to the participant's APOE epsilon4 status. Thus, phase 3 of the trial had separate protocols for APOE4 carriers, though non-carriers still had high rates or ARIA-E (14.2% in the highest dose group) [113].

Solanezumab is another humanized mAb targeting Aβ monomers without binding Aβ deposits and tested the idea that removing the soluble pool of Aβ would mobilize Aβ42 from plaques; this was subsequently proven in the phase 2 study [114]. However, phase 3 trial in mild AD showed non-significant slowing of cognitive decline, though the incidence of ARIA-E was low [115].

Gantenerumab, another mAb, bound Aβ fibrils and in mice, reduced Aβ plaques, prevented new plaque formation without altering plasma Aβ levels [116]. Clinical trials with the humanized antibody were terminated in phase 2/3 due to failure to reach primary endpoints [117].

Crenezumab, a mAb targeting multiple conformations of Aβ, with a higher affinity for oligomers vs. monomers [118]. This treatment has been shown to be relatively safe, but without significant benefits in phase 2 [119]. Phase 3 trials were launched, testing a higher dose of crenezumab for early AD, again not meeting the primary endpoints [120].

Aducanumab reacts with Aβ aggregates – both soluble oligomers and insoluble fibrils [121]. It had dose-dependent slowing of disease progression in phase 1b, though included high incidences of ARIA-E [122]. In March 2019, phase 3 trials were halted due to projected failure to reach primary endpoints [123].

BAN2401 bound and cleared Aβ protofibrils and was well-tolerated without ARIA-E incidences [124]. It is currently in phase 3 clinical trial after promising phase 2 results [125].

What can we learn from these trials? Passive immunization against Aβ tends to be ineffective when tested in AD patients with mild to moderate disease, which suggests that the therapy is simply administered too late in the disease. According to the amyloid cascade hypothesis, summarized in Fig, 1.2, Aβ accumulation occurs decades before cognitive symptoms appear, and triggers other events, such as tau hyperphosphorylation and aggregation, neuronal death and persistent neuroinflammation. These are then independent features, which will not be affected with any type of Aβ clearance, unless this therapy is used in a preventative manner. Figure 1.3 presents a summary of all current and abandoned anti-amyloid therapies.

1.6 BACE1 inhibitors

BACE1 is a prime therapeutic target in Alzheimer's disease due to this enzyme initiating the production of Aβ species. However, this enzyme has targets beyond APP, and the disruption of its function might lead to unintended side effects. To model inhibition of BACE1, several groups have generated BACE1 knockout mice using gene-targeting strategies. The $BACE1^{-/-}$ mice, though at first having reported to have no overt phenotype [126], have demonstrated axonal guiding defects [126-128], hypomyelination [129, 130], memory deficits[131], abnormalities in neurogenesis and astrogenesis [132], spine density reduction[133], schizophrenia endophenotypes [133], and seizures [134, 135]. Since these mice have had no functional BACE1 from the moment of conception, and for example, myelination is completed by adulthood [136], some of these phenotypes may not be part of side effects of BACE1 inhibition, though most would remain a concern. These knockout studies also suggest that BACE2, a homologue of BACE1, but

which has low neuronal expression, does not functionally compensate for BACE1 inhibition, and targeting BACE1 only to combat AD pathophysiology is therapeutically feasible.

Many BACE1 inhibitors also target BACE2 and have failed in human trials due to inability to display a benefit in symptomatic disease (lanabecestat, clinical trial NCT02783573, and verubecestat, clinical trial NCT01953601). Atabecestat trials have recently been discontinued due to side effects of elevated liver enzymes [137]. However, other BACE1 inhibitors are still in clinical trials, such as elenbecestat and CNP520 (clinical trials NCT03036280, and NCT03131453, respectively; see Fig. 1.3 for a summary of discontinued and active clinical trials of BACE1 inhibitors).

1.7 γ-secretase inhibitors and modulators

Because γ-secretase functions to generate Aβ, many therapies have been focusing on inhibition of the enzyme. Since γ-secretase is responsible for cleavage of a wide variety of targets, side-effects from GSIs are a great concern. In particular, the inhibition of Notch processing can lead to abnormal cell proliferation, which can explain the higher incidences of skin cancers in the treatment groups. To date, all γ-secretase inhibitors (GSIs) have failed in clinical trials (Fig. 1.3) [138]. Semagacestat, a GSI, was tested in mild-to-moderate AD patients, and phase 3 clinical trial resulted in worsening of cognition, as well as many adverse events such as gastrointestinal disorders, hepatocellular injury, skin rashes, hypersensitivity, and neoplasms [139, 140]. Avagacesat is another GSI that reached phase 2 clinical trials, reportedly having a higher selectivity for APP over Notch; however, it failed to slow dementia progression and has

higher incidences of diarrhea, nausea, skin rashes, squamous and basal cell carninomas [141]. In improved Notch assays, avagacestat been shown to be fairly non-selective and Notch-targeting [142].

In light of inefficacy and toxicity of the GSIs, a new approach is now considered. The first γ-secretase modulators were a subset of nonsteroidal anti-inflammatory drugs (NSAIDs) and could specifically lower Aβ42 levels and increase Aβ38 levels [143]. This cleavage pattern that generates less Aβ42 and more Aβ38 can be due to two possible mechanisms: induction of lower dissociation probability for the longer Aβ or increased processivity of the enzyme. Sulindac sulfide, ibuprofen, flurbiprofen and indomethacin modulate γ-secretase at 35-300 uM concentration. These GSMs were noncompetitive inhibitors of Aβ42 via binding to an allosteric binding site [144]. Unfortunately, further development of GSM NSAIDs was halted by dose-limiting toxicity. NSAID derivatives, such as tarenflurbil, have also failed in clinical trials due to poor CNS penetration and poor drug-like properties [145, 146].

Non-carboxylic acid imidazole-containing GSMs have been developed, and in particular, Eisai's E2212 reached phase 1 clinical trials and demonstrated safety, tolerability, and Aβ42 reduction in plasma [147]. Merck's pyrazolopyridine series demonstrate nanomolar in vivo efficacy in rat and monkey [148]. Currently, a phase I trial for NGP 555 is underway (NIH Project Number: 3R01AG049702-02S2), having so far shown no skin of GI toxicity.

This dissertation will describe the efficacy of a GSM, BPN-15606 (Fig. 1.4) *in vitro* and *in vivo* in the PSAPP model of AD at two stages of disease progression, pre-

plaque and post-plaque onset, and demonstrate that an anti-amyloid therapy must be administered before the onset of clinical symptoms to be effective.

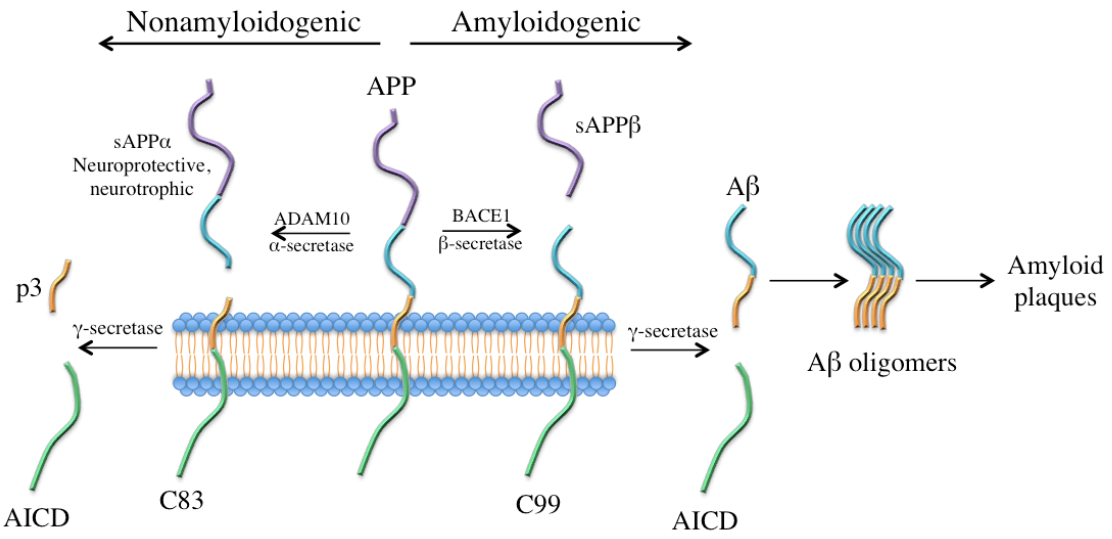

Figure 1.1. Two pathways of APP processing. In the nonamyloidogenic pathway, APP is cleaved by α-secretase (here, the metalloproteinase ADAM10 is shown). This generates sAPPalpha, which is neurotrophic and neuroprotective, and C83. The C83 fragment is then cleaved by γ-secretase to generate p3 and the AICD (APP intracellular domain). In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE1), which releases sAPPβ and C99. C99 is then cleaved by γ-secretase to generate Aβ and AICD. Aβ monomers can form soluble oligomers, which can then deposit into insoluble amyloid plaques. Adapted from Lichtenhaler and Haas, 2004 [149].

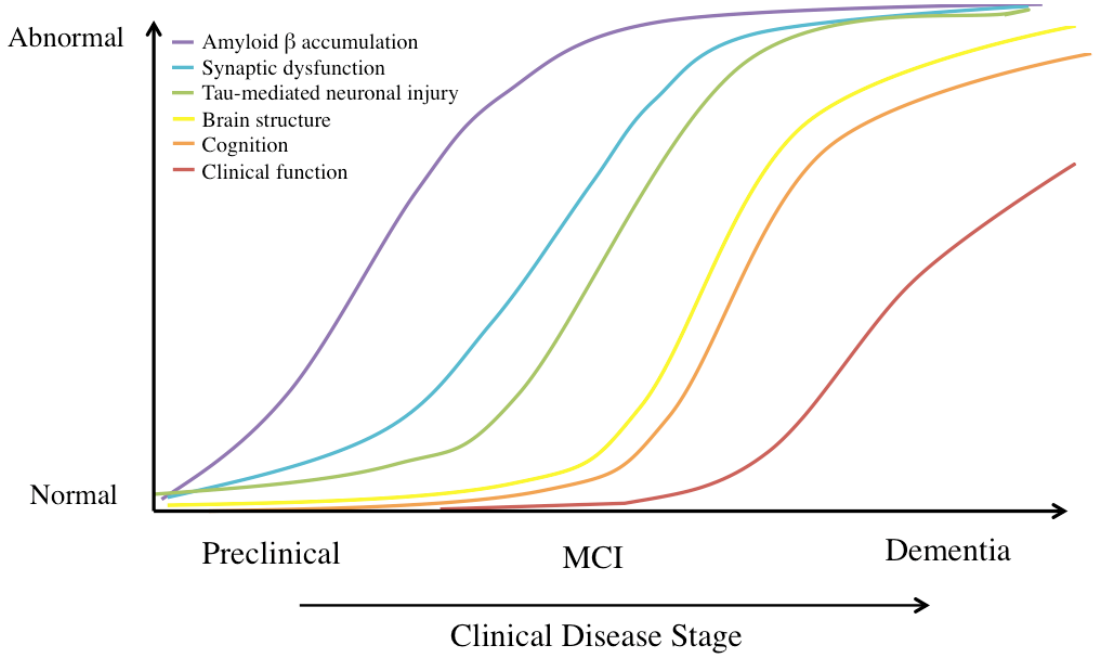

Figure 1.2. A hypothetical model of changes in biomarkers in AD. Amyloid β accumulation is measured by PET or CSF (cerebrospinal fluid) Aβ42 assay. It is followed by synaptic dysfunction, as assayed by functional MRI (magnetic resonance imaging) or FDG-PET (fluorodeoxyglucose positron emission tomography). Tau-mediated neuronal injury is measured via tau and phospho-tau levels in CSF. Brain structure alterations are measured by structural MRI. Cognition and clinical function are measures for clinical disease staging. Adapted from Sperling *et al*, 2011 [150].

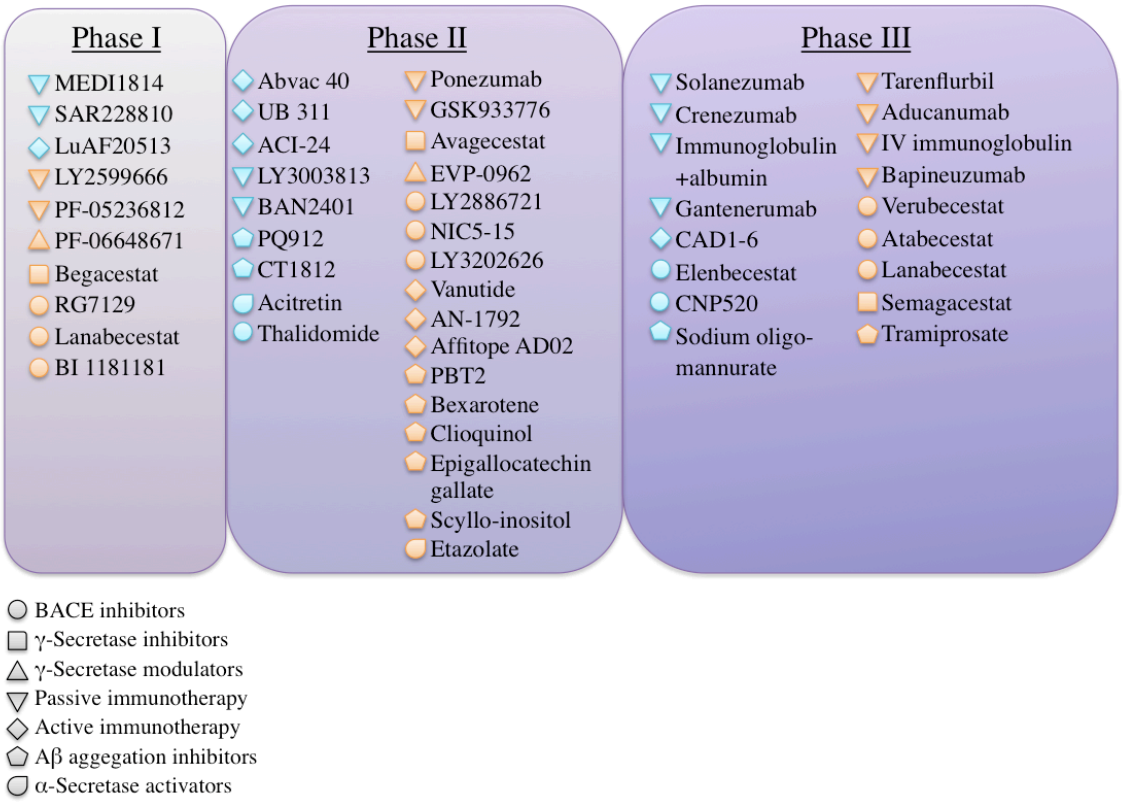

Figure 1.3. Current and abandoned anti-amyloid-β therapies in various stages of clinical development. Trials of therapies marked with orange are discontinued or inactive. Adapted from Panza et al, 2019 [151].

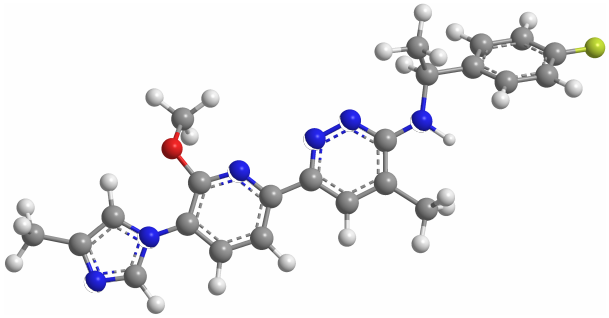

Figure 1.4. BPN-15606 structure. Color coding: nitrogens blue, oxygen red, fluorine yellow, carbon grey, hydrogen white.

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CHAPTER 2

The GSM BPN-15606 as a potential candidate for preventative therapy in AD

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PREFACE TO CHAPTER 2

Chapter 2 in full is an article submitted to Journal of Alzheimer's Disease. This work directly addresses the primary aim of this dissertation. The dissertation author was the primary investigator and author of this paper.

2.1 Abstract

Background: In the amyloid hypothesis of Alzheimer's disease (AD), the dysregulation of Aβ (amyloid-β protein) production and clearance leads to amyloid deposits, tau tangles, neuronal loss and cognitive dysfunction in AD. Thus far, therapies targeting the enzymes responsible for Aβ production have been found ineffective or having significant side effects.

Objective: To test whether a γ-secretase modulator, BPN15606, is an effective disease-modifying or preventative treatment in the PSAPP mouse model of AD.

Methods: We treated pre-plaque (3 month old) and post-plaque (6 month old) PSAPP AD transgenic mice for 3 months and examined behavioral, biochemical and pathological end points.

Results: BPN-15606 prevented cognitive impairment, reduced amyloid plaque load, microgliosis and astrogliosis associated with the AD phenotype of PSAPP mice when administered to pre-plaque (3 month old mice) but was ineffective when administered to post-plaque (6 month old mice). No treatment-related toxicity was observed.

Conclusion: BPN-15606 is a viable preventative therapeutic for AD, and is a candidate for early-phase human safety trials.

Keywords: Alzheimer's disease; BPN-15606; γ-secretase modulator; Cognitive deficits; Beta amyloid; PSAPP; preventative therapy

Abbreviations: AD, Alzheimer's disease. APP, amyloid precursor protein. Aβ, amyloid-β protein. GSM, γ-secretase modulator.

2.2 Introduction

Alzheimer's disease (AD) is a degenerative brain disease, and the most common form of dementia in the elderly, which affects about 5.5 million individuals in the US in 2017 (Alzheimer's Association Report). This number will only continue to grow as the proportion of population aged 65 years or older increases due to extending life span resulting from better medical care; currently, 10% of people in this group have AD. AD is the sixth leading cause of death in the US; deaths from this disease increased 89% from 2000 to 2014. Pathological alterations in the brain appear some 10-20 years prior to cognitive symptoms [1, 2]. The national cost of the disease was estimated to be $230 billion in 2017, which included significant unpaid time away from work for assisted care from friends and family of the patient, costs for providing long-term health care and for currently available palliative treatments which are limited to temporary and mild alleviation of cognitive and behavioral symptoms in a subset of patients [3, 4]. There is no cure, effective prevention or therapeutic regimens for altering the course of the underlying disease process [5].

The pathological features of AD include β-amyloid (Aβ) plaques, which accumulate extracellularly, and neurofibrillary tangles, which accumulate intracellularly, in numerous regions of the cerebral cortex and hippocampus in a staged temporal-spatial pattern [6]. The Aβ-containing neuritic plaques result from oligomerization of Aβ42, which is a product of cleavage of amyloid precursor protein (APP). APP can be processed through one of two distinct metabolic pathways, one of which is referred to as being "amyloidogenic" and produces Aβ40, the most abundant secreted Aβ peptide, as

well as the more fibrillogenic Aβ42, the major peptide found in both diffuse amyloid deposits and in compact neuritic plaques [5]. Familial Alzheimer's disease-linked mutations include those within the APP transmembrane domain, result in increased Aβ42/Aβ40 peptide level ratios (approximately two-fold), as well as missense mutations within PS1 and PS2 (presenilin 1 and 2), either of which harbor the catalytically active subunit of γ-secretase, the enzyme which ultimately proteolyzes APP to form Aβ peptides, similarly elevating the Aβ42/Aβ40 ratio two-fold[7-10].

The amyloidogenic processing of APP commences with cleavage by BACE1 (beta-site APP cleaving enzyme 1) which generates a soluble amino-terminal fragment sAPPβ and a membrane-bound carboxyl fragment APP-C99, also known an APP-CTFβ, which is further proteolyzed by γ-secretase, producing an AICD (APP intracellular domain) as well as a number of Aβ peptide variants including Aβ37, Aβ38, Aβ40, and Aβ42. Alternatively, APP can be processed through a non-amyloidogenic pathway initiated via α-secretase-mediated cleavage resulting in a soluble amino-terminal fragment termed sAPPα, along with a membrane bound APP-CTFα fragment, which is further processed by γ-secretase producing non-amyloidogenic products, including p3 and AICD [11].

Since Aβ generation requires the sequential proteolysis of APP by BACE1 and γ-secretase, these two aspartyl proteases have been thoroughly explored as therapeutic targets for AD. Many BACE1 inhibitors have failed human trials due to inability to display a benefit in symptomatic disease (lanabecestat, clinical trial NCT02783573, and verubecestat, clinical trial NCT01953601). Atabecestat trials have recently been

discontinued due to side effects of elevated liver enzymes [12]. However, other BACE1 inhibitors are still in clinical trials, such as elenbecestat and CNP520 (clinical trials NCT03036280, and NCT03131453, respectively). There also have been many failed γ-secretase inhibitor (GSI) programs. Semagacestat reached phase 3 clinical trials and was discontinued after patients exhibited worsening memory and increased risk of skin cancer possibly due to the inhibition of γ-secretase-mediated cleavage of Notch [13]. The following class of GSIs, dubbed "Notch-sparing" because of their reported selectivity for APP-CTF included begacestat [14] (discontinued for unstated reasons), and avagacestat (which was reported to cause cognitive worsening, along with gastrointestinal and dermatologic complications [15]).

Our approach of testing a γ-secretase modulator (GSM) instead of a GSI negates the issue of Notch-mediated side effects, as it preserves γ-secretase function and does not affect NICD (notch intracellular domain) generation via inhibition of epsilon-site proteolysis of Notch by γ-secretase. BPN-15606, selectively and potently attenuates Aβ42 and to a lesser extent Aβ40, while elevating the levels of non-pathogenic Aβ38 and Aβ37 [16].

γ-Secretase is composed of 4 proteins: presenilin (PS1 or PS2), nicastrin, Pen-2 and Aph-1. The enzyme is activated by Pen-2-mediated auto-proteolytic cleavage of PS1 or PS2 within the exon 9-coded segment. PS1 is cleaved to generate a PS1-NTF and a PS1-CTF, which form the catalytic center of the γ-secretase complex [17, 18]. Aph-1 and nicastrin assemble into a subcomplex, which then bind to the CTF of presenilin [19, 20]. Mutation of PS1 at Asp257 and Asp385 abolishes both the auto-proteolytic

(presenilinase) and γ-secretase activity; thus, it is the catalytic subunit of γ-secretase [21]. A PS1 mutant with the exon 9 domain excised (PS1ΔE9) is constitutively active and does not require the Pen-2-mediated cleavage [10, 22]. PSAPP, a transgenic mouse line that has this mutation, as well as the Swedish APP mutation, APP KM670/671NL, which enhances Aβ production, develops accelerated AD-like pathology, including plaque formation and cognitive deficits [23]. In addition, it exhibits synaptic abnormalities, disruption of neuronal connections and neuroinflammation [24-27]. We explored the extent of preventative or disease-modifying aspects of chronic administration of BPN-15606 using this mouse model to evaluate this γ-secretase modulator as potential therapy for Alzheimer's disease.

2.3 Methods

2.3.1 Compounds

The novel GSM BPN-15606, (S)-N-(1-(4-fluorophenyl)ethyl)-6-(6-methoxy-5-(4-methyl1H-imidazol-1-yl)pyridin-2-yl)-4-methylpyridazin-3-amine, was synthesized at Albany Molecular Research Institute (AMRI) Albany, NY using the methods reported in UCSD-MGH published patent application [28].

2.3.2 *In vitro* cell-based assay

The SH-SY5Y-APP cell line was derived by transfecting a human neuroblastoma (SH-SY5Y) cell line with a plasmid expressing wild-type human APP_{751} cDNA. 1 day prior to treatment, the cells were split at 75K/well in a 96 well plate. The next day, either vehicle, 2 concentrations of BPN-15606 (3.17 nM or 10 nM), or a GSI (81138, 199 nM) were added in triplicate. 24 hours post-treatment, media was collected and analyzed via

Meso Scale Discovery (MSD) 6E10 multiplex kit according to manufacturer's instructions. $A\beta_{total}$ levels were measured using a mAb-specific sandwich ELISA assay.b

2.3.3 Mice

B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax (PSAPP) from Jackson labs were used in this study at 3 or 6 months of age (referred to as pre-plaque and post-plaque groups, respectively). All mice were group housed with up to 5 mice per cage in standard 12-h light cycle, with free access to food and water. Mice were randomly assigned to treatment groups (n= 9-11 per group). All experimental procedures were reviewed and approved by IACUC at UC San Diego.

2.3.4 Pharmacological treatment and administration.

Gamma-secretase modulator BPN-15606 was administered for 3 months via gavage at 10mg/kg/day, 5 days a week. The drug solution was prepared weekly in 80% PEG400/0.1% Tween 20 (v/v) solution.

2.3.5 Behavior

All behavioral and locomotor tests were performed at the end of the treatment period while the mice were still being administered the treatments.

2.3.6 Total activity memory

The Total Activity Memory (TAM) test shows memory retention through habituation to the testing environment. An animal that remembers the testing environment will explore less than an animal with memory impairments. During the test, for the first 3 days, the animal was placed for 10 minutes into the Kinder Motor Monitor Cage rack system, which has a 7x15 beam configuration to monitor movement. The more

beams the animal breaks, the more the animal moves. There is a two-day break before Day 4, when the animal is placed into the same chamber for 10 minutes and its activity is analyzed. The first day of testing is also analyzed for spontaneous activity, which can be used as a baseline for the Morris water maze test.

2.3.7 Rotarod

In this performance task, the ability of a mouse to balance on a rotating rod at increasing speeds is measured over two days. There are 5 training trials on day 1, varying in speed, starting at 0 RPM ramping up to 10 RPM, then 0-20 RPM, and finally 0-40 RPM. Day 2 has 7 trials ramping to 40 RPM. The animals are allowed a minimum of 5-10 minutes rest between trials.

2.3.8 Morris water maze

The Morris water maze test was performed in room temperature water. Non-toxic Tempera poster paint is added to the water to increase the contrast between the water and the animal. The mouse was gently placed in the tank at water level, facing the pool wall, at one of the two start positions. The pool contained discrete cues on 4 inner sides, visible to the mice. A video camera was mounted on the ceiling directly above the pool and was used in conjunction with a videotracking system (AnyMaze by San Diego Instruments) to record the swim path of each mouse. The tracking program was launched as soon as the animal is in the water and stopped automatically once the mouse reaches the platform. If the animal cannot locate the platform in the water maze after 90 seconds, it will be immediately guided to it. This test is performed on the last 2 weeks of drug treatment. On Day 1, the animal is acclimated to the pool and shown where to locate the visible

platform to escape the water for four consecutive 90 sec trials, with a brief rest between trials starting at the same start location. On Days 2 and 3, the animal will be alternated between two starting points with the same visible escape platform and four 90 sec trials each with a brief rest. Days 4 and 5 consist of the same pattern of testing as Days 2 and 3, but with an invisible platform. The animals are then rested for 48 hours. Days 6 and 7 are identical to Days 4 and 5. On Day 8, the probe test is administered. The first 40 sec trial begins at the same start location as days two through seven, with the platform removed completely, the animal should remember where to go and stay in the vicinity. The second trial is also 40 seconds in duration, however the visible platform is placed and the animal starts at the second starting point. Animals that have memory impairments will demonstrate a greater latency to reach the platform location, as well as longer distance traveled. Mice, which failed to reach the platform in the visible probe trial, were excluded from further analyses.

2.3.9 Tissue and organ collection

The mice were sacrificed under isoflurane-induced anesthesia the day after the conclusion of behavioral testing. The brain was dissected and split into hemispheres. The right hemisphere was fixed in 4% PFA in PBS at 4°C for 24 hours, then placed in 30% sucrose until sectioning. Sections were cut coronally at 30 μm using a freeze-slide microtome, then placed into cryoprotectant (30% ethylene glycol, 30% glycerol, 40% 1x PBS) in -20°C until immunohistochemistry analysis. The left hemisphere was dissected into the hippocampus, cortex, and the rest of the brain, which were snap-frozen in liquid nitrogen for biochemical assays. Organs such as liver, GI tract, heart, thymus, lungs,

kidneys, spleen and spinal cord were fixed in 10% formalin and sent to Dr. Kent Osborn in the Pathology Core of the Animal Care Program Diagnostic Laboratory at UCSD for histopathology screening.

2.3.10 Biomarkers and pathology

Quantitations of Aβ38, Aβ40, and Aβ42 in brain extractions were determined using the Meso Scale Discovery (MSD) 4G8 multiplex kit according to manufacturer's instructions. $A\beta_{total}$ levels were measured using a mAb-specific sandwich ELISA assay [16]. Brain extractions were performed in RIPA Buffer (Cell Signaling) with the addition of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and PMSF. The samples were homogenized using IKA T8.01 Ultra Turrax Homogenizer, then spun at 14,000 g for 15 minutes at 4°C. The resulting supernatant was aliquoted and stored at -80°C. Formic acid extracts were prepared from RIPA-extracted pellets by homogenization in 70% cold formic acid on ice and centrifugation at 100,000g for 1 hour at 4°C. The supernatant was neutralized to pH 7.5-8 with 10 volumes of 2M Tris base, pH 11.5. The samples' protein concentration was measured via BCA assay (for RIPA-extracted samples) or Bradford Assay (for formic acid-extracted samples) and equal amounts of each sample were loaded onto MSD plates.

2.3.11 Immunofluorescence

To determine Aβ plaque load, serial coronal sections from PSAPP and wildtype mice (n = 5-6 per group) were washed in miliQ water, mounted on Fisherbrand Superfrost Plus microscope slides, and stained in 1% Thioflavin S solution. To support our findings, 82E1 (Clontech (10323, Takara Bio USA; 1:1000) staining was also

performed on free-floating sections. For assessment of neuronal and synaptic changes, sections were immunolabeled with antibodies against microtubule-associated protein-2 (MAP2) (MAB378, Millipore; 1:500) to label neuronal cell bodies and dendrites, GFAP (PA5-16291, Invitrogen; 1:250) and Iba1 (019-19741, Wako; 1:1500). Primary antibody staining was identified with fluorescently tagged secondary antibodies at 1:1000. Images were taken with Leica LiveImage fluorescence microscope at 10X using the same exposure across all images. Quantification of staining was performed double-blinded, using the ImageJ software from NIH. The threshold was adjusted consistently across all images. Particle count, area, and size were obtained using the Analyze Particles function in hippocampal or cortical regions of interest. The series of sections of each animal was averaged and grouped accordingly prior to statistical analysis. The number of series of section per animal ranged from 5-7 sections, and 3-5 animals were analyzed per group.

2.3.12 Toxicity

Formalin fixed tissues representing major organ systems were submitted to Dr. Kent Osborn, in the Pathology Core of the Animal Care Program Diagnostic Laboratory at UCSD, for light microscopic examination and assessment for abnormalities that might be related to the experimental treatment. The tissue included heart, aorta, lung, trachea, liver, gall bladder, kidney, spleen, thymus, spinal cord, vertebrae (bone), perivertebral muscle, bone marrow, esophagus, stomach, small intestine, cecum, colon and pancreas. Six micron paraffin-embedded, H&E-stained sections of these tissues from each animal were examined.

2.3.13 Statistical analysis

All experiments were performed blind coded. Values in the figures are expressed as means±S.E.M. To determine the statistical significance, values were compared using ANOVA or student's t-test. The differences were considered to be significant if *P*-values were <0.05. Data was analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

2.4 Results

2.4.1 *In vitro* cell-based assay

To determine the effect of the BPN-15606 on Aβ fragment and total Aβ secretion, we utilized a human neuroblastoma cell line, SH-SY5Y, expressing wild-type human APP_{751} cDNA. The cells were treated with either vehicle or 2 concentrations of BPN-15606 (3.17 nM or 10 nM). We have found that relative to baseline, BPN-15606 treatment significantly increased secreted levels of Aβ38 at 10 and 3.17 nM (Fig. 2.1A), decreased levels of Aβ40 at 10 nM (Fig. 2.1B), and decreased levels of Aβ40 at 10 and 3.17 nM (Fig. 2.1C) in cell culture supernatant, without altering the total levels of Aβ (Fig. 2.1D). These results along with previously published studies on this and related GSMs [16, 29] prompted us to pursue a comprehensive in vivo animal study.

2.4.2 Cognitive performance

We then investigated whether chronic administration of BPN-15606 led to improvements in learning and memory in a mouse model of AD. For this, we utilized 3- or 6-month old PSAPP mice (pre-plaque-formation and post-plaque-formation groups, respectively) and treated them 10 mg/kg/day BPN-15606 for 3 months. In the Morris water maze learning and memory task, BPN-15606-treated pre-plaque PSAPP mice

exhibited significantly reduced latency and distance to reach platform compared to vehicle-treated PSAPP mice (Fig. 2.2A and B). In fact, their performance was not significantly different from wild-type mice except during Day 4, where they were first introduced to the hidden platform; after Day 4, they performed at wild-type mouse levels. Post-plaque mice (6-month old at start of treatment) did not exhibit significantly different performance in distance (Fig. 2.2C) and latency (Fig. 2.2D) to platform compared to untreated transgenic mice. The rotarod performance task (Fig. S 2.1A and B) and total activity memory test (Fig. S 2.1C and D) did not reveal any significant differences in balance or activity between any of the groups. The results suggest that BPN-15606 administration led to better learning and memory in PSAPP mice during the learning paradigm.

2.4.3 Assessment of Aβ load

To determine whether chronic administration of BPN-15606 leads to reduction of plaque load in pre-plaque mice, we stained coronal brain slices with the 82E1 antibody, which recognizes soluble and fibrillar Aβ but not holo-APP (Fig. 2.3A and B). Immunohistochemistry revealed that BPN-15606 reduced the number of plaques relative to vehicle treatment in the cortex, but not in the hippocampus (Fig. 2.3C) in the pre-plaque mouse group. The average plaque size was also decreased in both the cortex and the hippocampus in the BPN-15606-treated pre-plaque mouse group as compared to vehicle-treated mouse group (Fig. 2.3D). In the post-plaque BPN15606-treated mice, there was no significant difference between the number (Fig. 2.3E) or size (Fig. 2.3F) of plaques compared to the vehicle-treated group.

2.4.4 Brain levels of Aβ

Next, we wanted to determine whether chronic administration of BPN-15606 leads to the increase of Aβ38, and reduction of Aβ40 and Aβ42 in the brain before and after plaque onset. To do this, we measured amyloid beta fragments in detergent-soluble and formic acid-soluble brain fractions in both vehicle-and BPN-15606-treated pre-plaque and post-plaque PSAPP mice. Detergent-soluble brain fractions of BPN-15606-treated pre-plaque mice contained significantly reduced levels of Aβ42 and a reduced Aβ42/Aβ40 ratio compared to the vehicle-treated group, while Aβ38 and Aβ40 levels remained unchanged (Fig. 2.4A). Formic-acid soluble brain fractions from BPN-15606-treated compared to vehicle-treated pre-plaque mice contain only slightly lower levels of all amyloid β fragments that did not reach statistical significance and exhibit an unchanged Aβ42/Aβ40 ratio (Fig. 2.4B). In the post-plaque treated mice, all amyloid beta fragment levels were similar in the detergent-soluble (Fig. S 2.2A) and formic-acid-extracted (Fig. S 2.2B) brain fractions, and the Aβ42/Aβ40 ratio was unchanged.

2.4.5 Microgliosis

In AD brain, Aβ deposits are often associated with microgliosis, the response of microglia to pathogenic insults, which is characterized by an increased number of activated microglia at the site of the insult [30]. It is also present in the PSAPP mouse model [27, 31]. We have assayed the degree of microgliosis through staining mouse brain slices with Iba1, ionized calcium binding adaptor molecule 1, which is a microglia- and macrophage-specific calcium binding protein [32]. Compared to wild-type mice, pre-plaque, vehicle-treated PSAPP mice exhibited a greater percentile of Iba1-positive area

and activated microglial counts in cortex (Fig. 2.5B and D) and hippocampus (Fig. 2.5C and E). BPN-15606 treatment significantly reduced the Iba1-positive area and the number of activated microglia in both brain regions. In post-plaque mice, BPN-15606 treatment did not reduce microgliosis in either the cortex (Fig. S 2.3B and D) or hippocampus (Fig. S 2.3C and E), which parallels our findings regarding its inability to reduce plaque load in this particular AD transgenic mouse aged group.

2.4.6 Astrogliosis

Astrogliosis is a common feature of neurodegenerative disease and is defined by an abnormal activation and increased numbers of astrocytes [33, 34]. The PSAPP mouse model also exhibits astrogliosis [27, 30, 31, 35]. To determine the effect of BPN-15606 on this aspect of neuroinflammation, we stained mouse brain slices for GFAP, using an antibody against glial fibrillary acidic protein, which is expressed in astrocytes [36]. We observed an elevation of GFAP-positive staining in PSAPP transgenic mice compared to wild-type mice, which was reduced in BPN-15606-treated mice (Fig. 2.6). Specifically, while GFAP-positive area was significantly higher in the cortex of the PSAPP transgenic mice compared to wild-type mice (Fig. 2.6B), BPN-15606 treated-mice have similar levels of GFAP to wild-type. We did not observe changes in GFAP-positive staining across groups in the hippocampus (Fig. 2.6C). Similarly, astrocyte size was increased in cortex and hippocampus of PSAPP transgenic mice, indicating astrocyte reactivity (Fig. 2.6D and E), and BPN-15606 treatment reduced astrocyte size to normal size, but only in the cortex. In contrast to this effect, the post-plaque PSAPP transgenic mouse group did not exhibit any benefit from BPN-15606 treatment, retaining an elevated GFAP-positive

area in the cortex compared to wild-type mice (Fig. S 2.4B and C) and displaying similar levels of GFAP in hippocampus as wild-type mice (Fig. S 2.4D and E).

2.4.7 Dendritic deficits

While dendritic deficits are a feature of AD and PSAPP mice [26], we did not observe significant differences between MAP2-positive staining in cortex or hippocampus of pre-plaque (Fig. S 2.5A and B) or post-plaque (Fig. S 2.5D and C) mice.

2.4.8 Toxicity

None of the treatments were associated with weight loss or a lack of weight gain (Fig. S 2.6). The analyzed tissues were all essentially normal, with no indication for apparent toxicity. Lesions that were noted are considered incidental, either as common background lesions in laboratory mice, secondary effects of euthanasia, or lesions in individual animals that, while not common, are not considered to be significant in the context of this study.

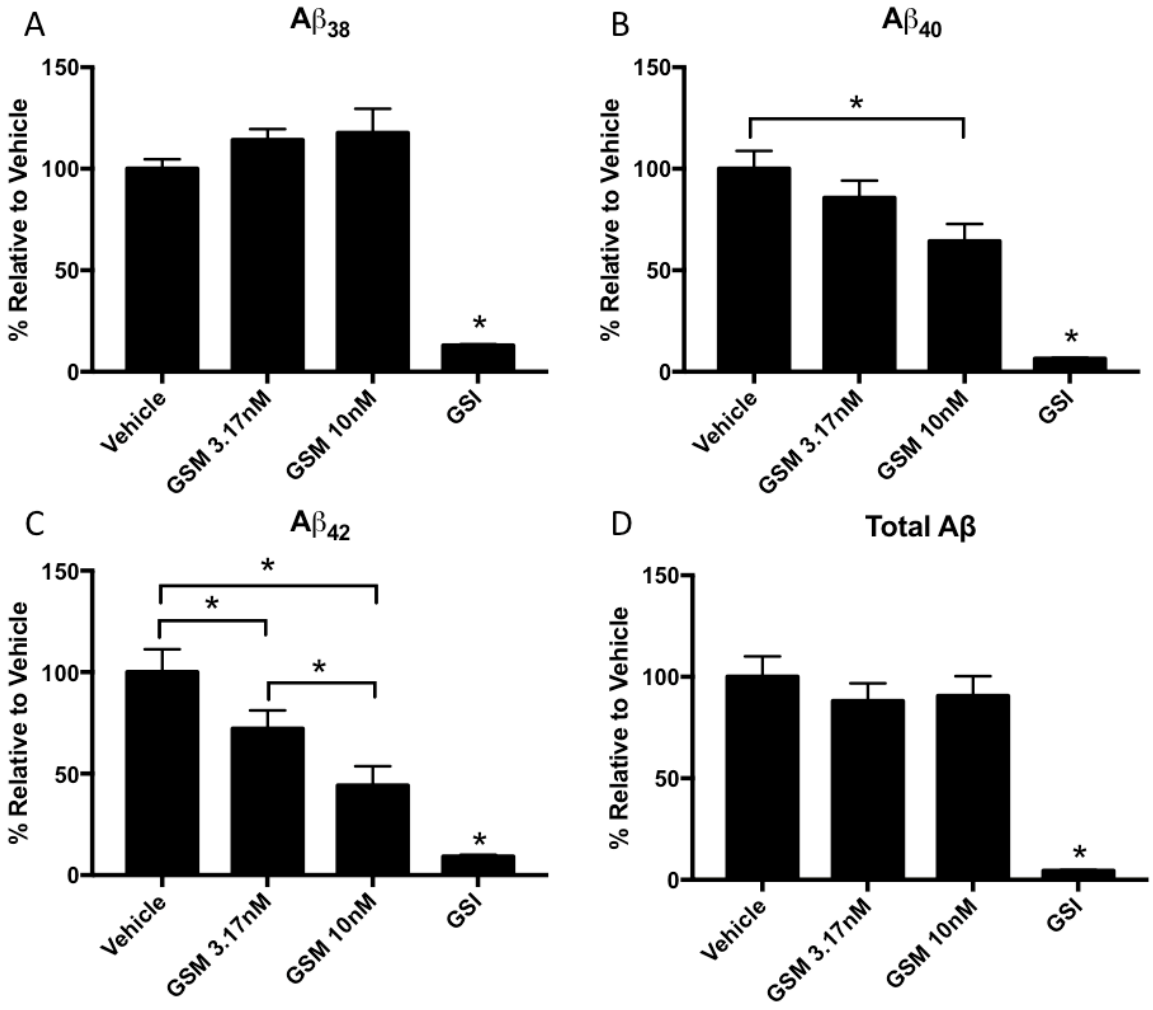

Figure 2.1. GSM treatment attenuates secretion of Aβ40 and Aβ42 species without affecting total Aβ. Levels of Aβ38 (A), Aβ40 (B), Aβ42(C) and total Aβ (D) in media of SHSY5Y-APP cells after 24 hour treatment with GSM, GSI or vehicle. All Aβ measurements utilized triplex MSD ELISA (n=3). Values presented as mean +/- SEM, normalized to vehicle.

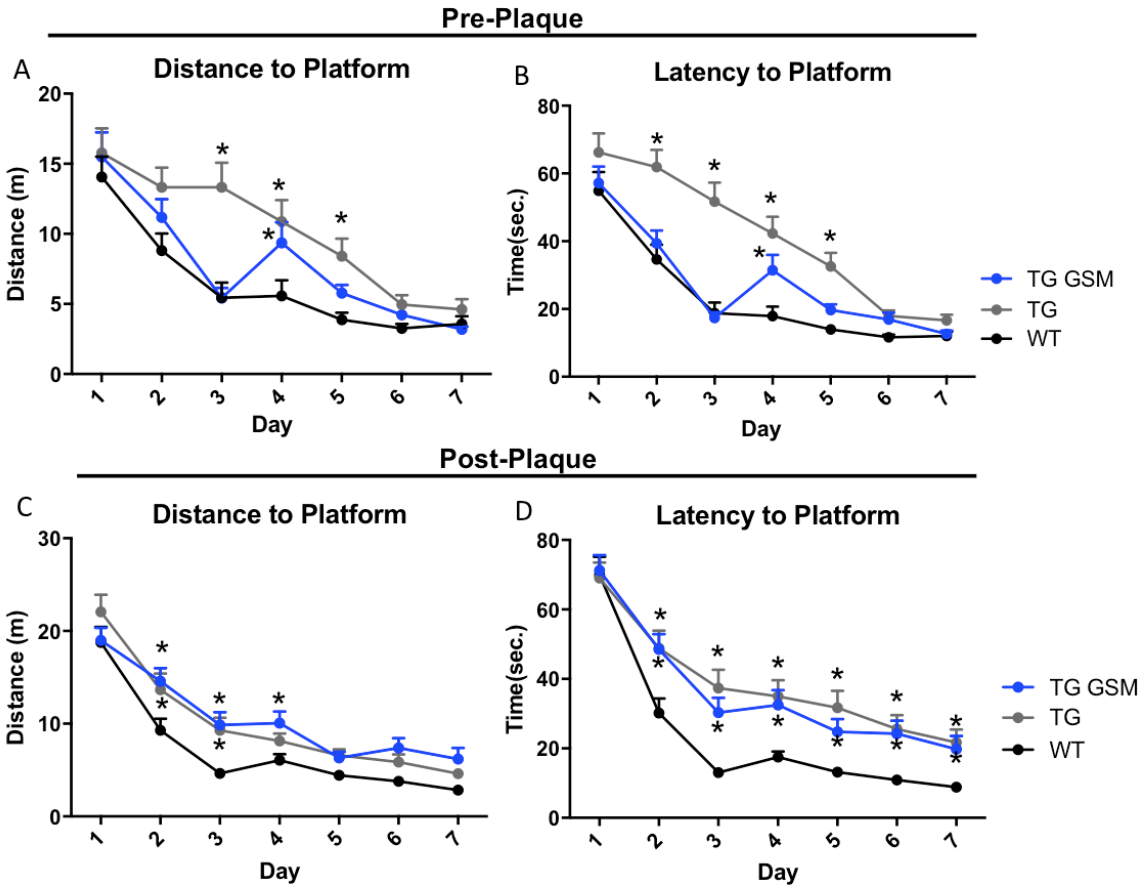

Figure 2.2. GSM treatment is correlated with enhanced performance in Morris water maze in 3 month old (pre-plaque) PSAPP (TG) mice. Pre-plaque GSM-treated mice (TG GSM) performed significantly better than vehicle-treated (TG) in the distance (A) and time (B) to platform measurements. Post-plaque GSM-treated TG mice performed as well as vehicle-treated TG mice in the distance (C) and time (D) to platform measurements, significantly worse than vehicle-treated wild-type mice (WT). N=9-10. Values presented as mean +/- SEM. *p>0.05, represents significant difference from WT.

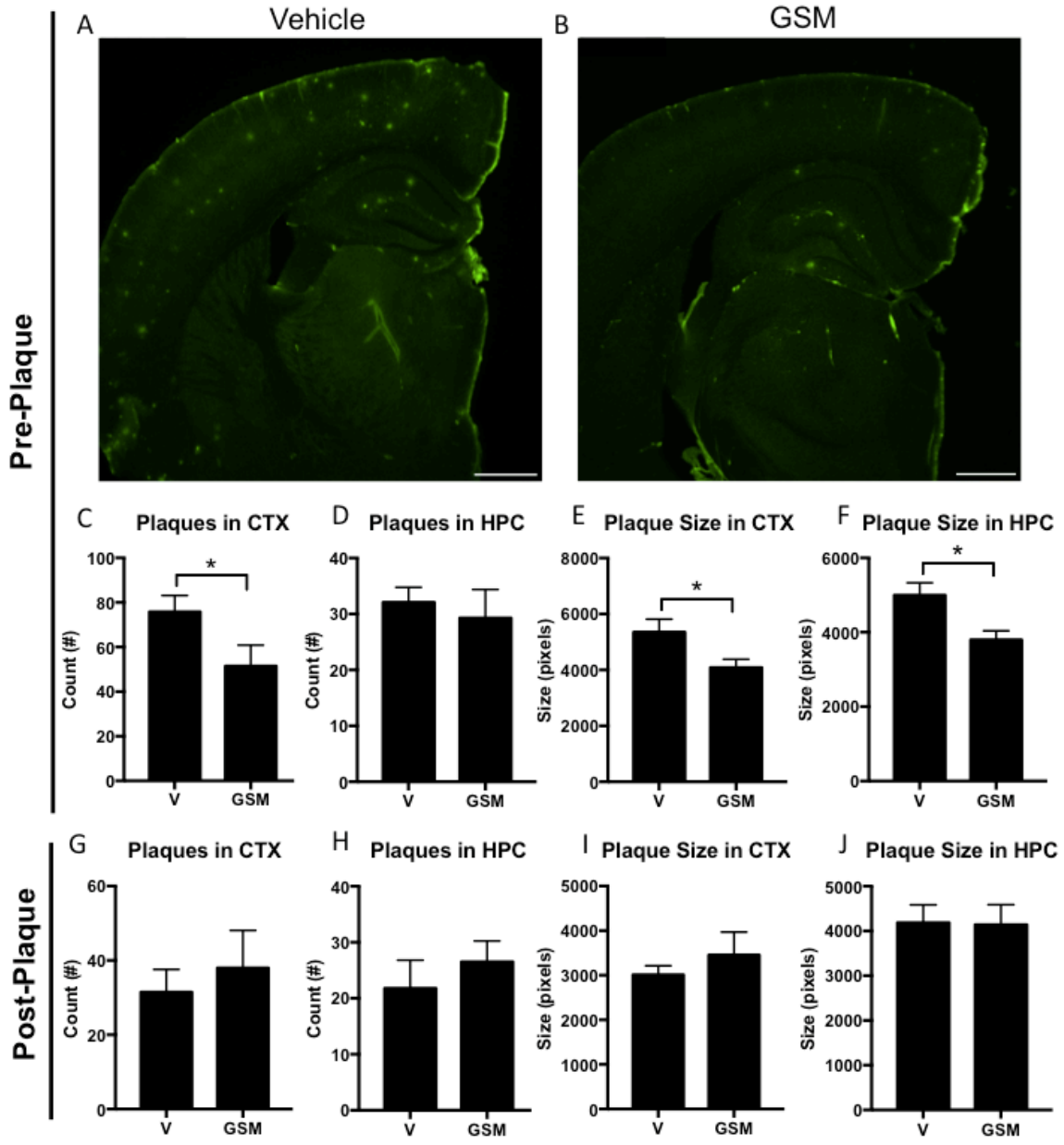

Figure 2.3. GSM treatment ameliorates Aβ plaque load and decreases the size and number of Aβ plaques in 3 month old (pre-plaque) PSAPP (TG) mice. Representative images of pre-plaque (A) vehicle- and (B) GSM-treated TG mice. 82E1 staining revealed that the number of plaques is decreased in cortex (C), and the average size of plaques in HPC and CTX (D) were significantly decreased in GSM-treated pre-plaque TG mice vs. vehicle-treated pre-plaque TG mice. Scale bar, 1 mm. No differences in number (E) or size (F) of plaques were observed the brains of GSM-treated post-plaque TG mice as compared to vehicle-treated post-plaque TG mice. n=5. Values presented as mean +/- SEM. $p<0.05$.

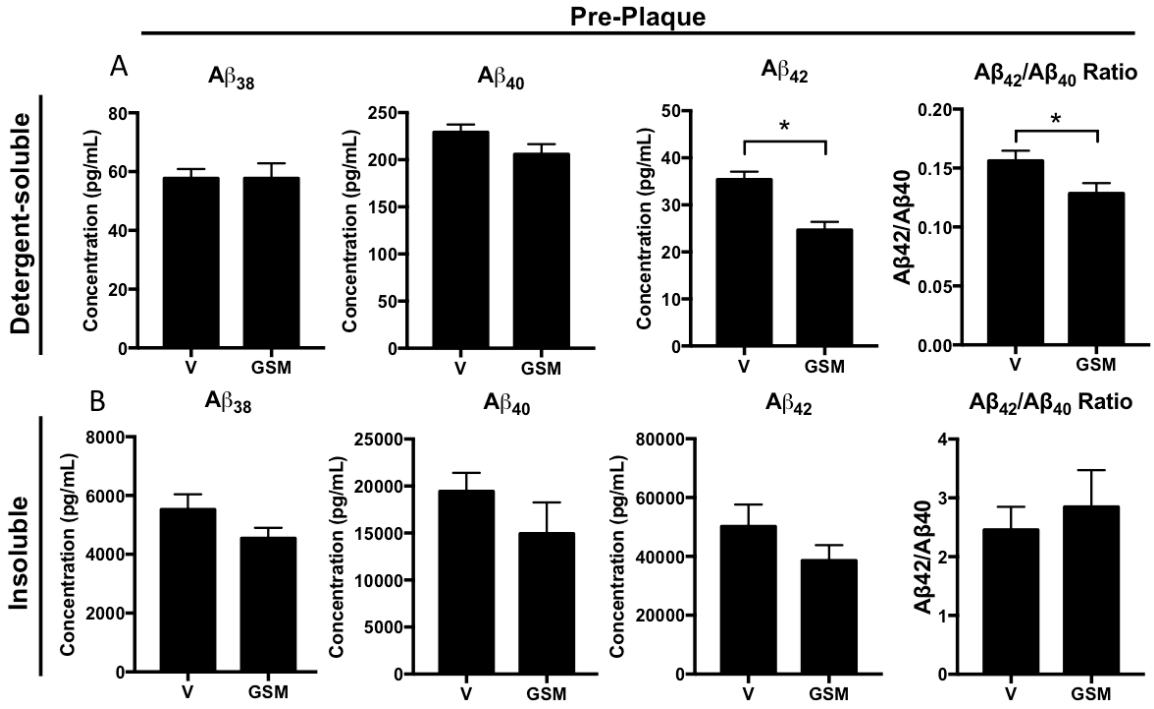

Figure 2.4. GSM treatment reduces detergent-soluble Aβ42 and Aβ42/Aβ40 ratio in the cortex of pre-plaque PSAPP mice. (A) RIPA-extracted brain fractions from pre-plaque GSM-treated PSAPP mice have significantly less Aβ42 and a reduced Aβ42/Aβ40 ratio compared to pre-plaque vehicle-treated PSAPP mice. (B) Formic-acid soluble brain fractions show no significant differences between treated and untreated groups. Values are expressed as mean ± SEM. N=9-10. $p<0.05$.

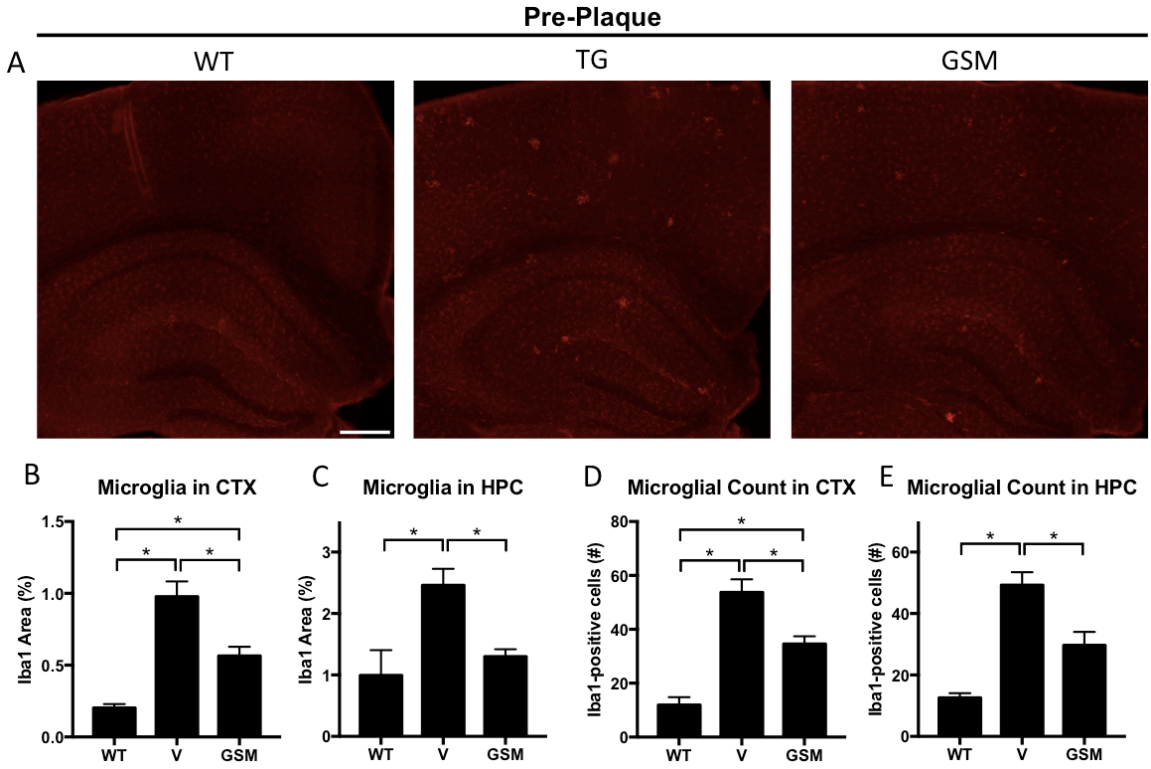

Figure 2.5. GSM treatment reduces microgliosis in pre-plaque PSAPP mice. Representative images of Iba1 stain (A). Scale bar represents 500 um. BPN-15606 treatment reduces percentage of Iba1-positive area in cortex (B) and hippocampus (C) of pre-plaque PSAPP mice compared to vehicle-treated pre-plaque PSAPP mice. Microglial count is also reduced after BPN-15606 treatment in cortex (D) and hippocampus (E) compared to vehicle. Values are expressed as mean ± SEM. n=4-5. $p<0.05$.

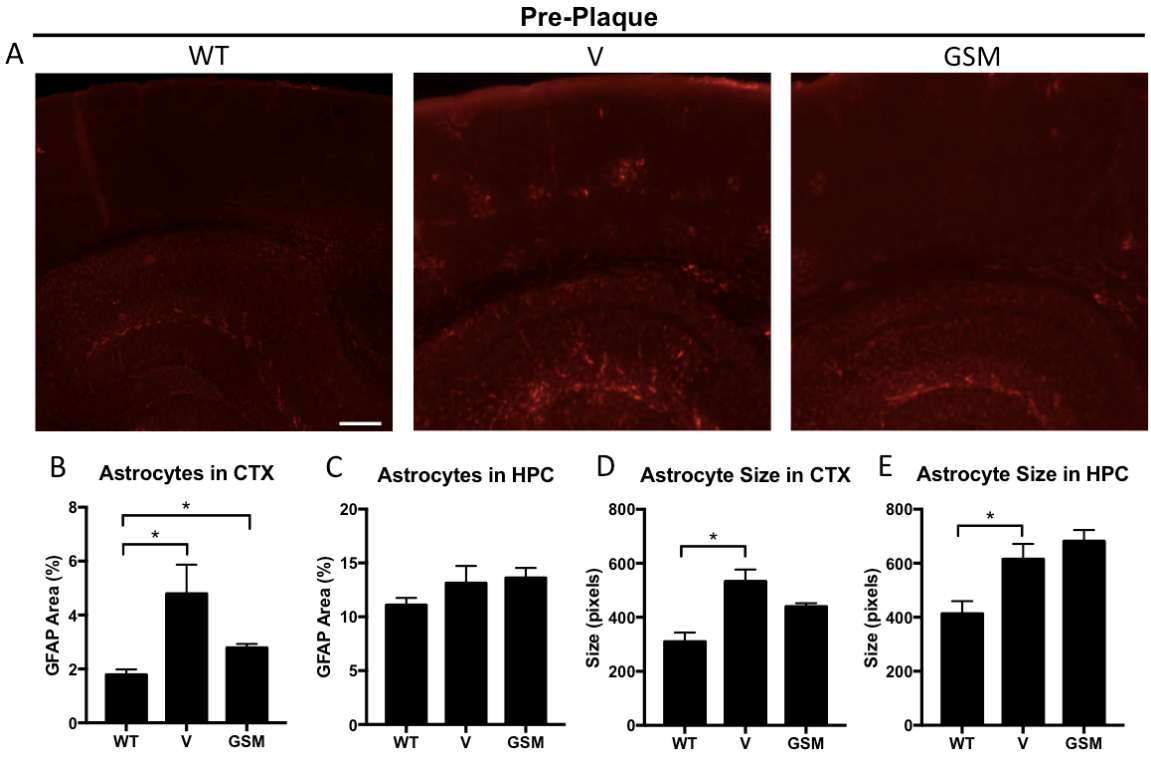

Figure 2.6. GSM reduces astrogliosis in the cortex of pre-plaque PSAPP mice. (A) Representative images of GFAP staining in cortex and hippocampus of pre-plaque PSAPP mice. Scale bar represents 500 um. (B) Percentage of GFAP-positive area in cortex and (C) hippocampus. (D) Average size of individual astrocytes in cortex and (E) hippocampus. Values are expressed as mean ± SEM. n=4-5. $p<0.05$.

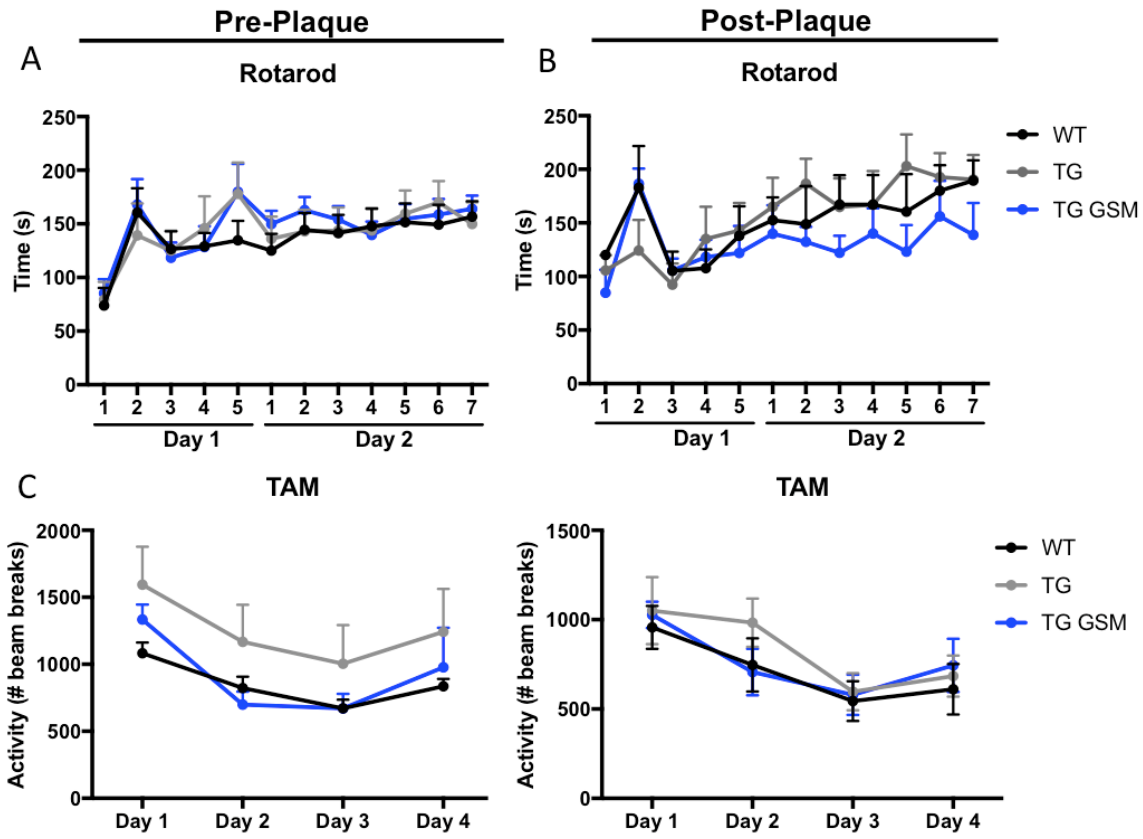

Figure S 2.1. Motor activity is not significantly different among all groups. The rotarod performance test indicated no balancing abnormalities among pre-plaque (A) and post-plaque (B) mouse groups, and an upward general trend of time spent on the rotating beam. TAM (total activity memory) test shows pre-plaque (C) and post-plaque (D) groups habituating to the testing environment and decreasing exploratory activities without significant inter-group differences. Data are mean +/- SEM. n=9-11.

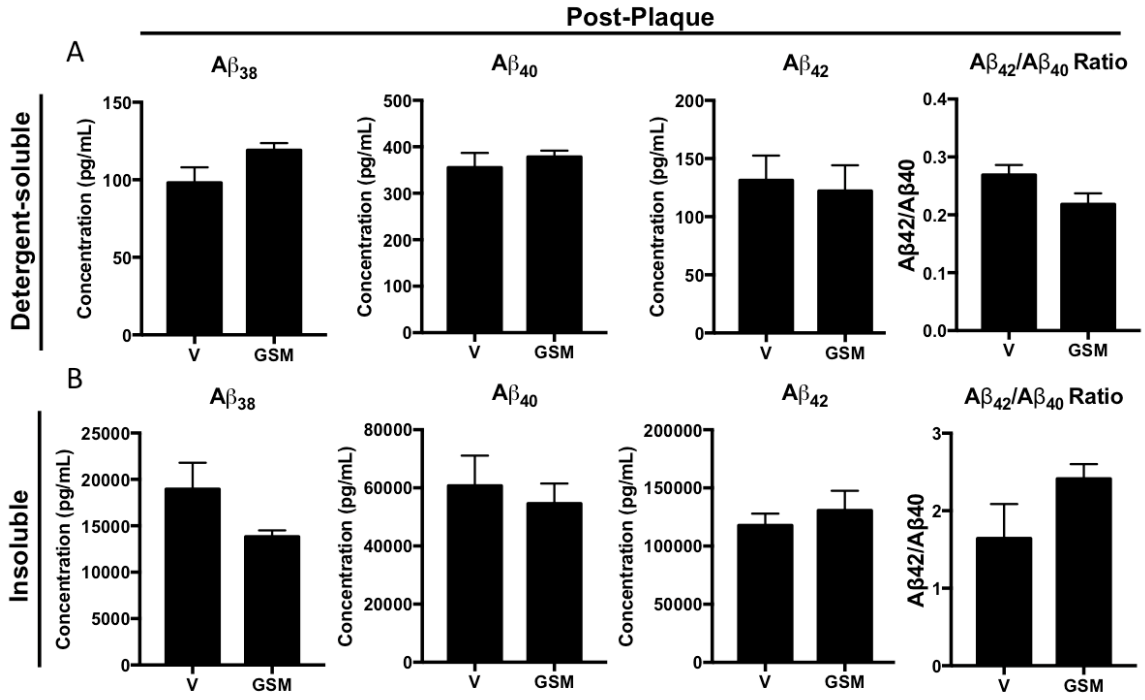

Figure S 2.2. GSM treatment does not affect Aβ fragment levels in post-plaque PSAPP mice. (A) RIPA-extracted and (B) formic acid-extracted brain fractions from GSM-treated post-plaque PSAPP mice do not show differences in Aβ fragment levels compared to vehicle-treated mice. Values are expressed as mean ± SEM.

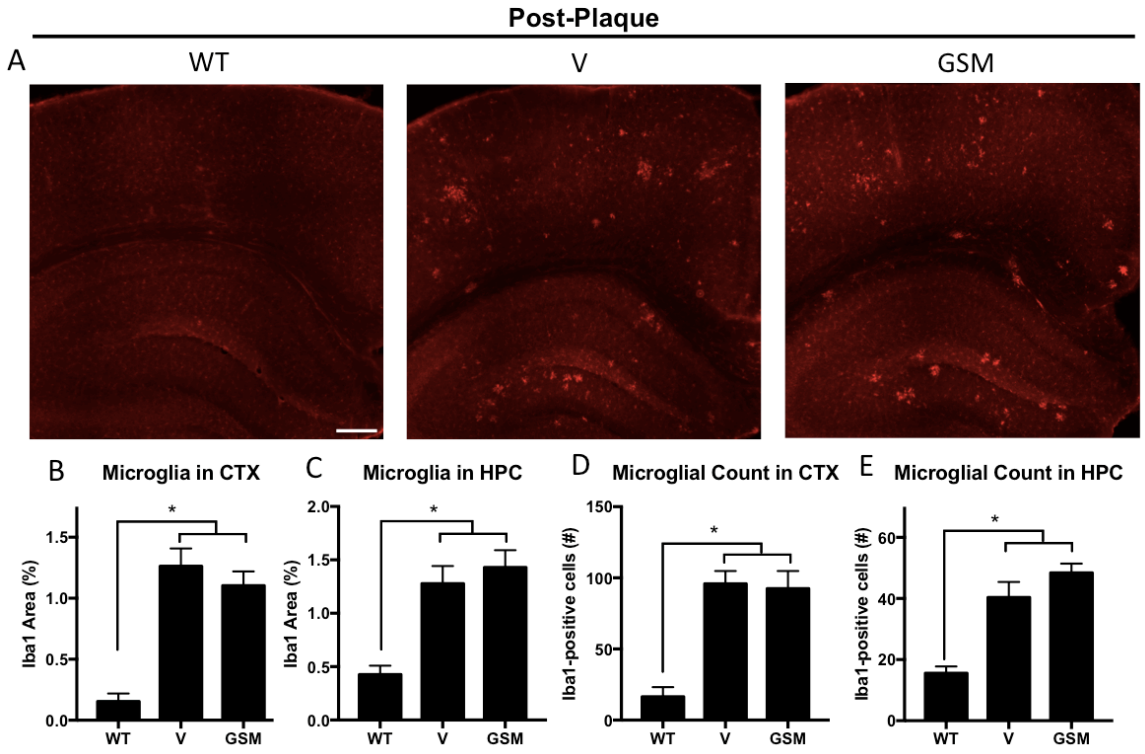

Figure S 2.3. GSM treatment does not reduce microgliosis in post-plaque PSAPP mice. Representative images of Iba1 stain (A). Scale bar represents 500 um. BPN-15606 treatment has no effect on percentage of Iba1-positive area in cortex (B) and hippocampus (C) of post-plaque PSAPP mice compared to vehicle-treatment. Microglial count was unchanged after BPN-15606 treatment in cortex (D) and hippocampus (E) compared to vehicle. Values are expressed as mean ± SEM. n=4-5. $p<0.05$.

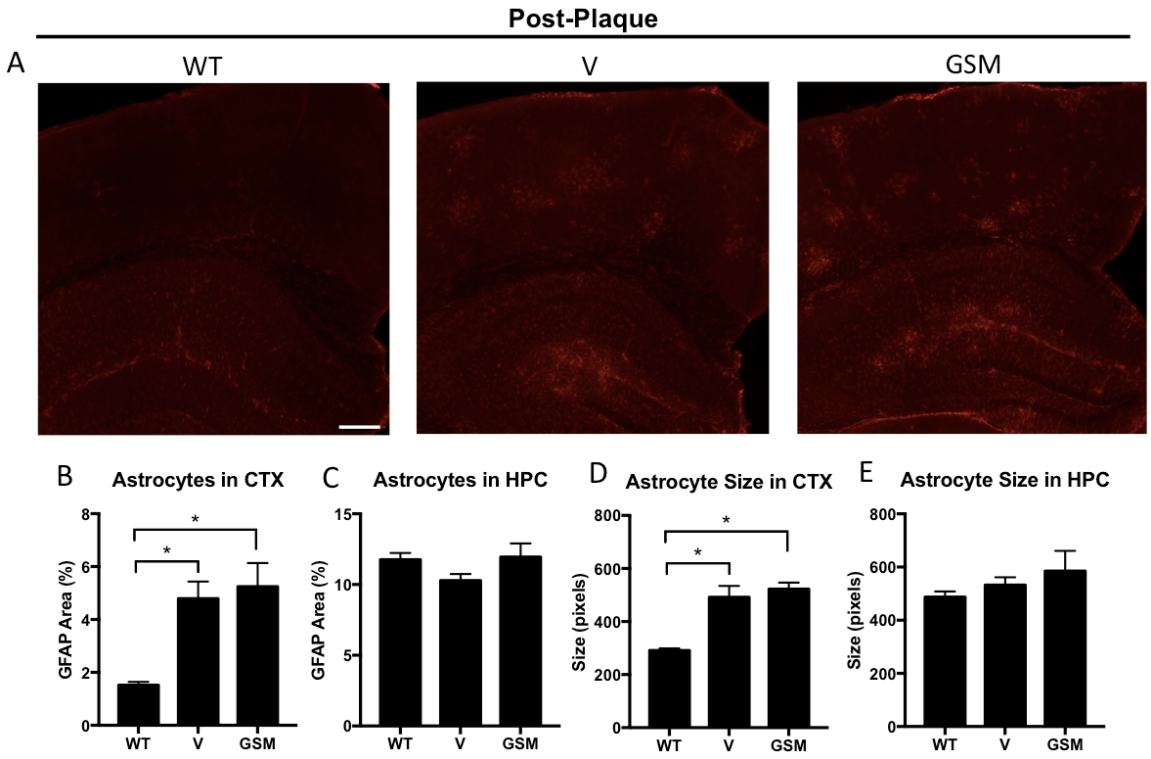

Figure S 2.4. GSM has no effect on astrogliosis in post-plaque PSAPP mice. (A) Representative images of GFAP staining in wild-type, vehicle- and GSM-treated post-plaque PSAPP mice. BPN-15606-treated and untreated transgenic mice had elevated GFAP-positive area in cortex (B) but not hippocampus (C) compared to wild-type mice. Astrocytes were enlarged in BPN-15606-treated and vehicle-treated post-plaque PSAPP transgenic mice cortices (D) but not hippocampi (E) compared to wild-type mice. Scale bar represents 500 um. Values are expressed as mean ± SEM. n=4-5. $p<0.05$.

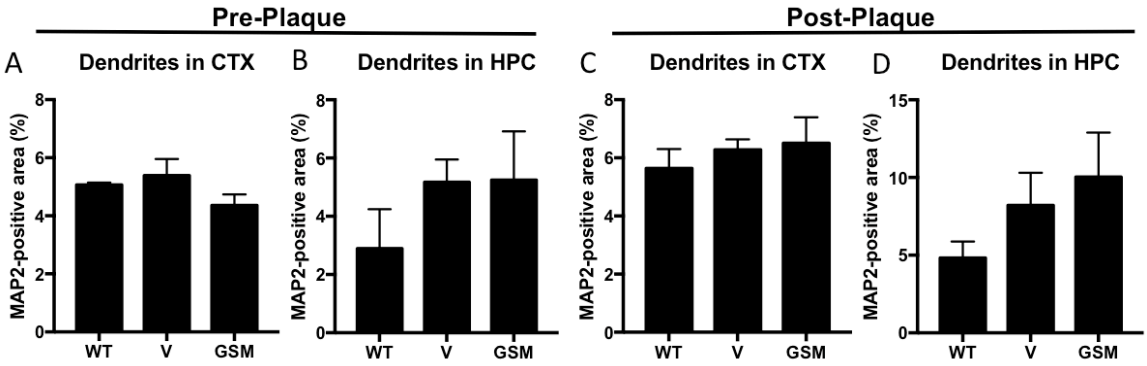

Figure S 2.5. Dendritic differences between wild-type and PSAPP mice were not observed. Optical density measurements of MAP2-positive cells in cortex (A) and hippocampus (B) of pre-plaque PSAPP mice, and in the cortex (C) and hippocampus (D) of post-plaque PSAPP mice were not significantly different between wild-type, vehicle- or GSM-treated PSAPP mice. Values are expressed as mean ± SEM. n=4-5.

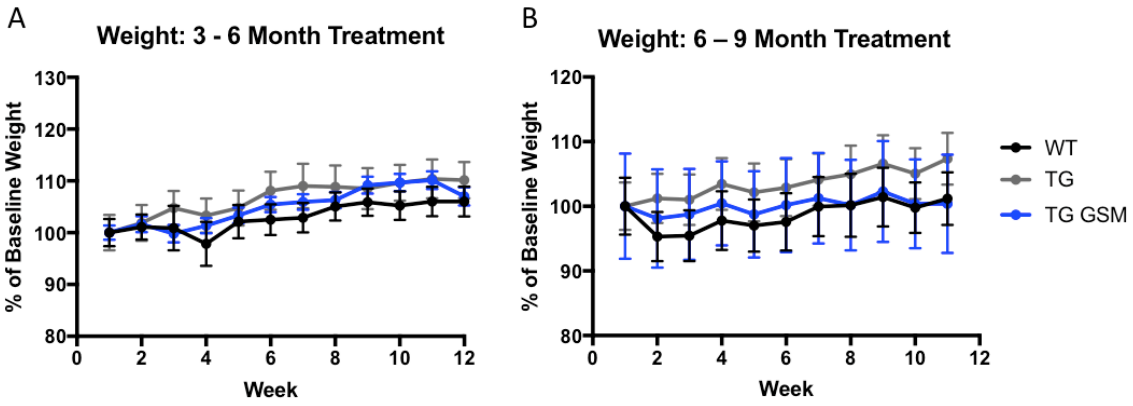

Figure S 2.6. Weekly group averaged body weights of PSAPP mice. Pre-plaque PSAPP mice 3-6 months of age (A) and post-plaque PSAPP mice, 6-9 months of age (B) fed ad libitum normal mouse chow shown as % of baseline. Data are mean +/- SEM. n=9-11.

2.5 Discussion

This is the first study to comprehensively address the efficacy of a 3-month oral treatment with a potent γ-secretase modulator, BPN-15606, on behavioral, pathological and biochemical features of AD in the PSAPP mouse model at pre-plaque and the post-plaque ages. We have found that *in vitro*, BPN-15606 treatment increases levels of secreted Aβ38, and decreases the levels of Aβ40 and Aβ42. The longer forms of β-amyloid are more hydrophobic and fibrillogenic, and are the primary components of amyloid plaques [37]. Thus, BPN-15606 shifts APP processing to more benign β-amyloid species, attenuating deposition of plaques.

We also show an effective intervention in disease progression when BPN-15606 is administered to PSAPP mice before plaque onset. In behavioral tasks, BPN-15606-treated mice performed similarly to age-matched wild-type mice. These cognitive benefits were correlated with reduced plaque pathology as evidenced by immunohistochemistry. Biochemically, the level of detergent-soluble Aβ42 fragments and Aβ42/Aβ40 ratio was decreased in the cortex of pre-plaque mice. Insoluble Aβ42 levels showed a trend toward reduction after BPN-15606 treatment, though it was not statistically significant at the dose administered. The theory that alterations in the Aβ42/Aβ40 ratio play a critical role in AD pathogenesis has been supported by multiple studies [38]. With the reduction of plaques, we also saw reduced inflammation in terms of microgliosis and area of astrogliosis in the cortex, with a trend towards reduction in size of astrocytes after treatment. In the hippocampus, microgliosis, but not astrogliosis, was ameliorated by the BPN-15606 treatment. Our immunohistochemical data shows that

the astrocytes in the brains of the transgenic mice are uniformly present throughout the hippocampus, unlike in the cortex, where they clump around plaque locations (Fig. 2.4A). Since BPN-15606 does not directly affect neuroinflammation to our knowledge, but rather indirectly ameliorates it through the reduction in plaques, non-plaque associated inflammation will therefore not be reduced with treatment. These results lead us to conclude that BPN-15606 is an effective preventative treatment in this transgenic mouse model.

Given that 6-month old mice already have plaques, and that other memory tests, plaque measurements, and Aβ fragment levels in cortex did not reflect an effect of the treatment, BPN-15606 does not appear to have a disease-modifying effect in amyloid-laden mice. The reason why the brain amyloid β measurements do not reflect any treatment-based differences is likely because of the fact that at 9 months, the plaque deposition in the brains of PSAPP mice is severe, and further alterations in APP processing will not result in significant effects.

In light of multiple failures of γ-secretase-targeting therapies, which mostly focus on inhibition, we propose an alternative method of modulating the activity of γ-secretase via BPN-15606 or one of its analogues. Given that γ-secretase has close to 90 different type 1 membrane protein potential substrates, which marks its involvement in multiple cell processes such as cell fate determination, neurite outgrowth, cell adhesion and migration and synapse formation [39, 40], it is important to consider the enormous potential for side effects for any therapy that targets γ-secretase. Using BPN-15606, we did not detect any treatment-associated abnormalities following a thorough toxicological

analysis. Additionally, taking into account the substantial evidence that Aβ has important physiological functions, such as antimicrobial/antiviral activity [41, 42] and synapse regulation [40], aiming for blanket attenuation of all Aβ species may have multiple deleterious consequences. The data presented in this paper demonstrates efficacy and safety of the γ-secretase modulator BPN-15606 treatment on AD-like pathophysiology in PSAPP transgenic mice, only when used in a preventative manner (prior to significant Aβ plaque deposition). These results may help to explain, at least in part, the lack of efficacy of numerous anti-amyloid-based therapeutics which have thus far been administered primarily, if not exclusively, to subjects already presenting with evidence of significant cerebral amyloidosis [43-45].

2.6 Acknowledgments

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privately held company (Neurogenetic Pharmaceuticals) that holds rights to a γ-secretase modulator, distinct from the GSM BPN-15606 described herein, currently in clinical development. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Chapter 2, in full, has been submitted for publication of the material as it may appear in Journal of Alzheimer's Disease. **Prikhodko O**, Rynearson K, Sekhon T, Nguyen P, Rissman RA, Tanzi RE, Wagner SL. BPN-15606 as preventative therapy for Alzheimer's disease. 2019. The dissertation author was the primary investigator and author of this paper.

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CHAPTER 3

Conclusions and Future Directions

3.1 Summary of findings

This dissertation shows that the GSM BPN-15606 is a viable preventative therapy for AD. In cell culture studies, BPN-15606 administration decreased the secreted levels of Aβ40 and Aβ42, which are the more pathogenic species of Aβ. We also found that when BPN-15606 is administered to PSAPP mice before plaque onset, it is an effective intervention in disease progression. The cognitive performance of the treated PSAPP mice was similar to wild-type littermates. Their plaque pathology and detergent-soluble Aβ42 was also reduced compared to untreated PSAPP mice. With the reduction of plaques, we also saw reduced inflammation in terms of microgliosis and area of astrogliosis in the cortex. The administration of BPN-15606 to post-plaque formation PSAPP mice did not result in any of the benefits that were seen post-treatment in the younger pre-plaque PSAPP mouse group, suggesting that BPN15606 does not have a disease-modifying effect when administered after significant pathology has occurred. In both the younger and the older mouse group, the treatment was well-tolerated and did not show any apparent toxicity.

3.2 Ongoing and future studies

Our therapy is focused on modulating the activity of γ-secretase, and our GSM has been shown to be Notch-cleavage-sparing; other γ-secretase targets that are cleaved at the epsilon cleavage site, like Notch, must also be spared. Though the GSM treatment

did not exhibit any overt off-target effects, further studies can look into the specific effect on various γ-secretase targets.

Although we have recently isolated and characterized a recombinantly expressed human wild-type enzyme and a clickable photo-affinity benzophenone analog in order to map the BPN-15606 binding site, we do not yet know its location on PS1-NTF. AD-associated mutations in PS1 may change the binding of the GSM and alter the efficacy of the compound for affected patients. This could be tested in patient-derived iPSC lines that have mutations in PS1 in a manner similar to our cell-based assay.

3.3 Concluding remarks

Many questions remain unanswered in the vast field that is Alzheimer's disease. Although targeting pathologic Aβ as the primary event in AD pathology is the aim of many anti-AD therapies, targets that are further along in disease progression may provide suitable options for combination therapy. Pathologic tau is one such target; physiologically, its role is to stabilize microtubules, which are components of the cytoskeleton that are involved in mitosis, cell motility, and intracellular transport [1]. Pathologically, hyperphosphorylated tau disengages from microtubules, misfolds, aggregates, causes axonal transport dysfunction and neurodegeneration [2]. It is also self-propagating, and present in considerable amounts by the time of AD diagnosis based on clinical symptoms [2, 3]. Effective therapeutics targeting tau, co-administered with anti-amyloid approaches and others described below, could slow or stop the progression of AD.

Neuroinflammation also plays a big factor in AD pathology. Though the inflammatory response is intended to be a protective function, persistent inflammation results in synaptic impairment, inhibition of neurogenesis, neurodegeneration and increase in APP synthesis [4, 5]. Developing a strategy to combat neuroinflammation is imperative to stopping the cycle of inflammation leading to neurodegeneration and must be part of the administered regimen for an AD patient.

An even more complex task is encouraging neuroregeneration in a brain with significant neuronal loss; repairing neuronal connections that have been lost would be an important road to recovery from AD at any state of the disease. However, aberrant neurogenesis must also be avoided, as epilepsy and further cognitive decline are potential side effects [6].

Most of the data collected on therapies in both preclinical animal studies and in clinical trials suggest that preventative treatment would be most effective [7]; to do this, we must have a cost-effective, reliable, minimally invasive technique to detect AD onset before clinical symptoms appear. As the imbalance of amyloid clearance and production is thought to initiate AD pathology, research into blood-based biomarkers in particular has shown correlation between the ratios of APP/Aβ42, Aβ42/Aβ40, and brain amyloid β burden [8]. Reliable biomarkers are also crucial for measuring the outcomes of clinical trials and may better inform our future approaches to curing this disease.

Limited proof-of-concept trials with patients who have well-studied familial early-onset AD mutations can be utilized to test and develop better therapies for AD. After all, this approach led to the development of statins to treat coronary artery disease

and stroke: in 1981, Mabuchi et al. have demonstrated the effect of 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor, compactin, in 7 patients with familial hypercholesterolemia caused by a mutation in the low-density lipoprotein (LDL) receptor gene. Compactin was shown to reduce LDL cholesterol levels without affecting high-density lipoprotein cholesterol. This study led to the development of statins, a related class of molecules, which reduce the incidence of coronary artery disease and stroke by more than 25% according to the American Heart Association. This model of proof-of-concept trial can be utilized in AD with carriers of familial AD-linked mutations: because we understand the progression of the disease in these patients, we can start treatment at an appropriate stage. This dissertation concretely demonstrates that treating AD with an amyloid β-targeted therapy, such as a GSM, before clinical symptoms appear, is more effective rather than administering the drug after the clinical symptoms have manifested. This should inform further therapy development for this unmet medical need, and patients that carry familial AD-linked mutations with a well-characterized disease course can provide a turning point in our fight against AD.

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