Investigating the Role of the Amyloid Precursor Protein in the Pathogenesis of Alzheimer's Disease

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ABSTRACT

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Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder characterized by a progressive loss of cognition. Histopathologically, AD is defined by the presence of two lesions, senile plaques (SP) and neurofibrillary tangles (NFT), which result from the accumulation and deposition of the amyloid-β peptide (Aβ) and the aggregation of hyperphosphorylated tau protein, respectively. Aβ is formed upon sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases and is secreted extracellularly. The accumulation of extracellular Aβ is thought to initiate a pathogenic cascade resulting in synaptic dysfunction in neurons, followed by the their eventual demise through apoptosis. However, while Aβ has been shown to be increased in AD patients' brains, little is known about how the cleavage of APP and the subsequent generation of Aβ is influenced or if the cleavage process changes over time. Moreover, while the effects of Aβ on neurons are known, the exact mechanism remains unclear. Many have postulated that Aβ exerts its effects by binding a putative receptor, but the search for an Aβ receptor has so far remained inconclusive. Interestingly, one of the proposed potential receptor for Aβ is APP itself. In this model, soluble oligomeric Aβ binds cell-surface APP, inducing its dimerization leading to all the downstream effects of Aβ in cells -- e.g. cell death and/or synaptic dysfunction.

Moreover, it has been proposed that Aβ can promote its own production in neurons, thereby initiating a pathogenic loop. However, isolating Aβ-induced APP signaling has remained challenging due to the promiscuous nature of Aβ binding. To work around this problem, we used an antibody-mediated approach to artificially trigger the dimerization of cell-surface APP in cells. We found that dimerization of APP could recapitulate all of the effects of oligomeric Aβ in hippocampal neurons, triggering neuronal death at high concentrations and interfering with normal synaptic functions low concentrations. We also found that dimerization of APP is sufficient to promote the amyloidogenic pathway, by increasing levels of the β-secretase BACE1, resulting in increased Aβ production. Finally, we found that dimerization of APP triggered caspase-dependent cleavage of APP and the formation of a second neurotoxic fragment, termed C31, which also mimics the effects of Aβ in hippocampal neurons. Taken together, our data provides support for the occurrence of a positive pathogenic feedback loop involving Aβ, APP and C31 in neurons.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease-domain protein
ADP	adenosine diphosphate
AICD	APP intracellular domain
APH-1	anterior pharynx-defective 1
APOE	lipid-transporting protein, apolipoprotein E
APP	amyloid precursor protein
APLP1	APP-like protein 1
APLP2	APP-like protein 2
ARF	ADP ribosylation factor
Aβ	β-amyloid
BACE1	β-site of APP cleaving enzyme
C31	31 amino acid long peptide resulting from caspase-cleavage of APP
C100	APP fragment resulting from β-secretase-mediated cleavage
C83	APP fragment resulting from α-secretase-mediated cleavage
cAMP	3'-5'-cyclic adenosine monophosphate
CREB	cAMP-response element-binding protein
CR1	complement receptor 1
CLU	clustrin gene
CT	carboxyl terminus of APP, containing 100 or 83 amino acid residues
DMSO	dimethyl sulfoxyde
EEA1	early endosome antigen 1
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK1	extracellular signal-regulated kinase 1

GAG	glycosylamineglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GGA	golgi-localized, gamma adaptin ear-containing, ARF-binding protein
hAPP	human APP
HSPG	heparan sulfate proteoglycans
IDE	insulin degrading enzyme
IgG	immunoglobulin G
KPI	Kunitz class of protease inhibitor
LTD	long term depression
LTP	long term potentiation
MoAPP	mouse APP
NFT	neurofibrillary tangle
PCR	polymerase chain reaction
PEN-2	presenilin enhancer 2
PICALM	phosphatidylinositol binding clathrin assembly protein
PS1	presenilin-1
PS2	presenilin-2
qPCR	quantitative PCR
RNA	ribonucleic acid
sAPPα	soluble fragment of APP after α-secretase cleavage
sAPPβ	soluble fragment of APP after β-secretase cleavage
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering RNA
SP	senile plaque
STS	staurosporine
TGN	trans-golgi network

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In loving memory of my wonderful parents,

Guy and Colette Blanchet

CHAPTER 1: GENERAL INTRODUCTION

1.1 Neuropathology of Alzheimer's disease (AD)

1.1.1 Historical perspective

In 1901, German psychiatrist and neuropathologist Alois Alzheimer was presented with the case of a 51-year old woman named Auguste Deter at the Frankfurt Asylum. The patient exhibited a peculiar cluster of behavioral symptoms. He described her as being cognitively impaired and suffering from auditory hallucinations, delusions, paranoia and a near complete loss of short-term memory and comprehension. Alzheimer continued to follow Auguste Deter until her death on April 8th 1906. Shortly after, he requested that her records and brain be sent to Munich, where he undertook a series of studies on the neuropathological features of her illness. In his description of the histopathological findings of the disease he noted the following: "In the center of an otherwise almost normal cell there stands out one or several fibrils due to their characteristic thickness and peculiar impregnability." He also chronicled the presence of abundant plaque-like features: "Numerous small miliary foci are found in the superior layers. They are determined by the storage of a peculiar material in the cortex" (Alzheimer, 1906). What Alzheimer described in his findings are what are now commonly referred to as neurofibrillary tangles (NFT) and senile plaques (SP), which are the hallmark brain lesions in the disease that would eventually bear his name, Alzheimer's disease (AD) (reviewed by Maurer et al., 1997).

1.1.2   Epidemiology

Today, over a century after it was first described, AD represents one of the most serious and costly health issues not only in the United States, but worldwide. It is the most common type of dementia, accounting for an estimated 60-80% of all dementia cases (Maslow, 2010). In 2007, according to the national statistics from the Aging, Demographics and Memory Study (ADAMS; Plassman et al., 2007), there were well over 2 million people over the age of 71 afflicted with the disease (Table 1-1), a number that is projected to reach 7.7 million people by 2030 and to increase to as many as 16 million people by 2050 by some estimates (Hebert et al., 2003). Across gender, 7% and 11.5% of men and women respectively over the age of 71 have the disease (Figure 1-1). In terms of mortality, AD was the fifth leading cause of death in the United States in 2006 for those older than 65 years old (Heron et al., 2009). In fact, while other major causes of death have been on a steady decline over the past few years, those for AD have continued to rise (Figure 1-2).

Age (years)	All Dementia	AD
71-79	712,000 (375,000-1,050,000)	332,000 (181,000-483,000)
80-89	1,996,000 (1,590,000-2,401,000)	1,493,000 (1,111,000-1,875,000)
≥90	699,000 (476,000-922,000)	556,000 (348,000-763,000)
Total	3,407,000 (2,793,000-4,021,000)	2,381,000 (1,849,000-2,913,000)

95% CI in parentheses

Table 1-1. National estimates of the number of individuals with dementia or AD by age categories

National statistics from the Aging, Demographics, and Memory Study (ADAMS). (Created from data from Plassman et al., 2007)

Figure 1-1. Estimated percentage of Americans aged 71+ with dementia by gender
National statistics from the Aging, Demographics, and Memory Study (ADAMS, 2002).
(Created from data from Plassman et al., 2007)
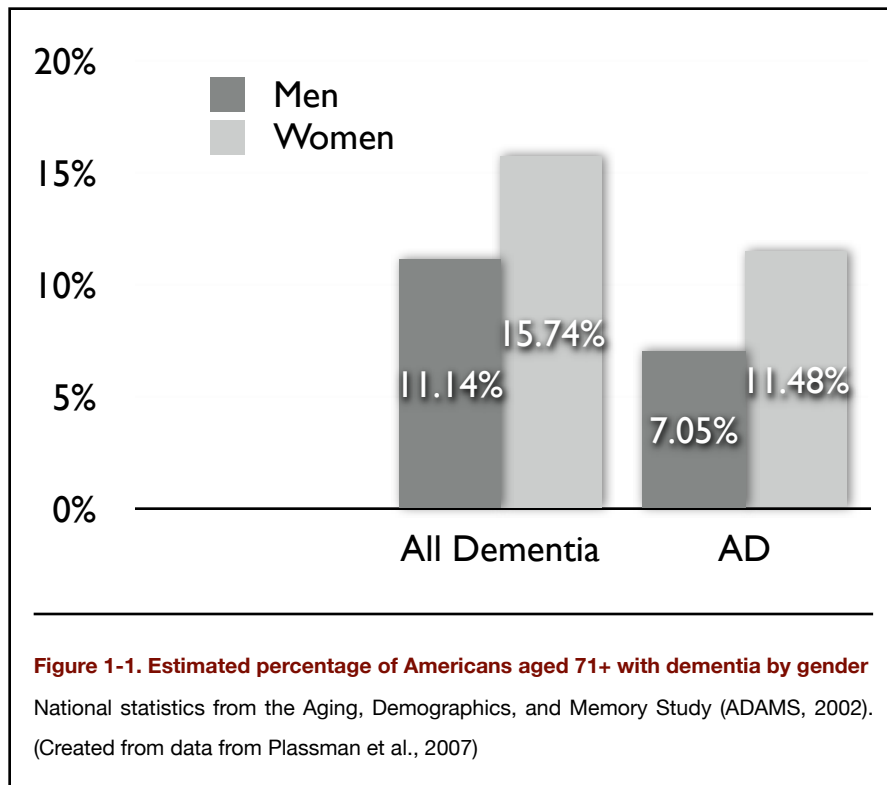
Unfortunately, it is unlikely that these numbers will reverse course in the near future. Current therapeutic approaches have yielded modest results at best. One possible reason for the lack of success may be due to the fact that reliably diagnosing AD continues to pose a significant challenge, especially in its early stages. Consequently, it is possible that by the time a patient is diagnosed with AD, it may be too late to reverse or even slow down the tremendous damage already inflicted on a patient's brain and cognitive functions over several years of living with the disease. Hopefully, a thorough understanding of the neuropathological features of AD may help in the development of better diagnostic tools, allowing for more precise and earlier recognition of abnormalities.

Figure 1-2. Percentage changes in selected causes of death between 2000[a] and 2006[b]

National Center for Health Statistics; Deaths: Final Data for 2000 [a](Minino et al., 2002) and 2006 [b](Heron et al., 2009). (adapted from Maslow, 2010)
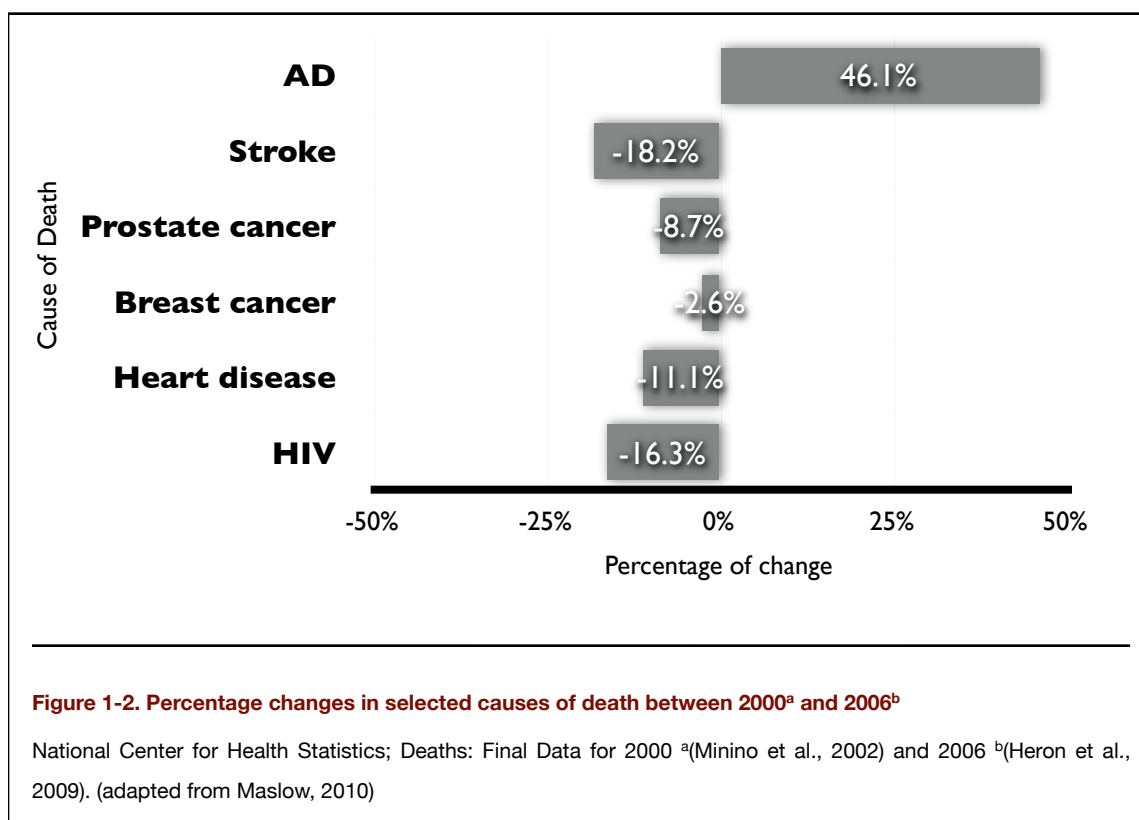
1.1.3 Clinical features of AD

The nature of the trigger(s) that kick-starts the Alzheimer's disease process has so far remained poorly understood. It is believed however, that the damage to the brain may begin several years before any overt cognitive problems. As the damage becomes more pronounced, the disease slowly progresses through various stages, as outlined in Figure 1-3 (reviewed by Forstl and Kurz, 1999), typically reflective of the underlying neuropathology (discussed later). Memory problems are usually the first noticeable symptoms, developing from seemingly simple forgetfulness to a more pervasive loss of recent memory. A meticulous neuropsychological evaluation at this pre-clinical stage may reveal that these individuals suffer from with a condition called amnestic mild cognitive impairment (MCI). Sufferers of MCI have noticeably more memory problems

than normal people of the same age, but their symptoms are not as severe as those with AD. Most, but not all people with MCI, eventually enter the mild dementia stages, where they experience a significant impairment in learning and memory, sometimes accompanied by some aphasic or visuoconstructional deficits. As a result, these patients may have difficulties in communicating, as their vocabulary and word fluency diminishes and may even experience some spatial disorientation. They may also experience a reduced ability to plan, judge and organize, especially in complex tasks. Usually, patients in the mild stages are able to survive on their own without much supervision. Over time, their symptoms worsen as they enter the moderate stages of AD.

Logical reasoning, planning and organizing abilities significantly deteriorate during this stage, as do their language, reading and writing skills. Patients may lose their ability to recognize familiar faces and some may even experience visual hallucinations. Behavioral changes, such as sudden violent physical and verbal outbursts, or excessive passivity are also common in these patients. At this stage, they usually cannot function properly without close supervision and will require even closer attention as they progress into the advanced stages of AD.

In the advanced stages of AD, most cognitive functions are severely impaired. Early biographical memories are often lost and language skills are typically reduced to simple phrases or even single words, leaving patients unable to articulate even their simplest needs. Restlessness and aggressive reactions become more frequent, as they often misinterpret or misunderstand their caregiver's interventions. They can also experience other motor disturbances, leading to rigidity or displays of primitive reflexes, such as snouting and grasping reactions. Eventually, patients experience a deterioration of their musculature and mobility, forcing them to become bedridden and unable to feed

themselves. Pneumonia, followed by myocardial infarction and septicemia are the most frequent terminal events in AD.

Figure 1-3. Progression of AD, from MCI to the advanced stages and a description of the typical symptoms associated with each stage
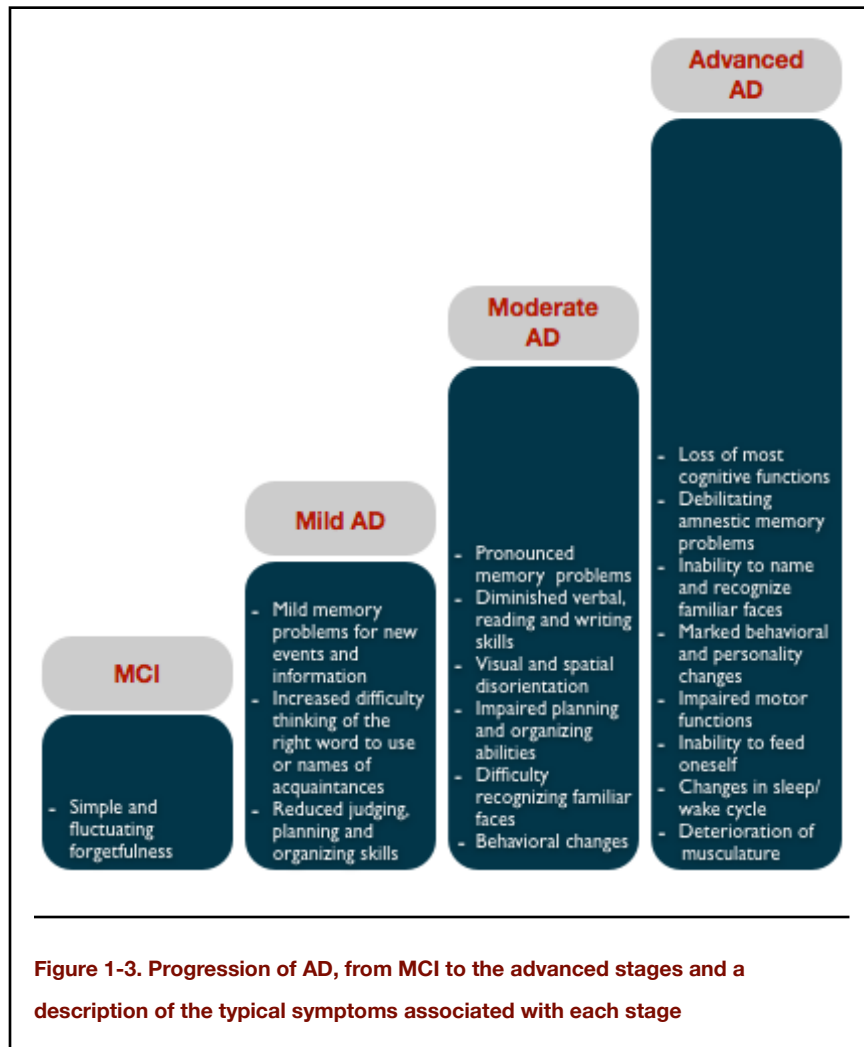
1.1.4   Macroscopic changes in AD brain

Upon examination at the autopsy table, the brain from an AD patient reveals numerous clues that could be considered useful for diagnostic purposes. However, most gross visible changes are usually non-specific, as there is a significant overlap with those seen in specimens taken from cognitively normal older individuals during the course of

normal aging (reviewed by Perl, 2010). In most AD patients, a close examination of the brain reveals variable degrees of frontal and temporal cortical and sub-cortical atrophy (Figure 1-4B), reflected by severe thinning of the cortical gyri and widening of the sulci (Figure 1-4C). In most cases, primary motor, sensory and visual areas of the brain are usually spared from such atrophy. Although atrophy and loss of overall brain weight is usually associated with normal aging (Figure 1-4A), there may be an additive effect in AD patients compared to aged-matched controls. However, the degree of brain weight loss overlaps significantly among both groups (Terry, 1986), making it almost impossible to use brain weight and cortical thickness as a determinants of AD. It should be noted that when examining early-onset or familial cases of AD (onset before the age of 65), a clear and obvious difference emerges when comparing the degree of atrophy and loss of brain weight observed in those patients with their aged-matched cognitively normal counterparts (Ridha et al., 2006; Schott et al., 2003).

Finally, AD cases almost always present with significant atrophy of the amygdala (Unger et al., 1991) and of the entorhinal cortex and hippocampus, with an associated dilation of the adjacent temporal horn of the lateral ventricle (Frisoni et al., 1999). In fact, both of these regions are thought to be the first ones affected in the early stages of AD (Braak and Braak, 1985, 1991). Such findings in an autopsy, when accompanied with clinical data and with sufficient microscopic evidence (e.g. amyloid deposits and neurofibrillary tangles), usually provide a strong indication of an AD case. Alternatively, the lack of these microscopic findings is an indication that other factors are at the root of the dementia.

Figure 1-4. Gross macroscopic brain changes in AD

(**A**) 79-year-old cognitively normal patient's brain showing a moderate amount of cortical atrophy. (**B**) An 89-year-old AD patient's brain showing an unusually high level of cortical atrophy. (**C**) AD brain showing severe atrophy of the frontal, temporal and parietal lobes atrophy, widening of the sulci and narrowing of the gyri. (Images courtesy of Dr. Jean-Paul Vonsattel; Columbia University, NY)

1.1.5 Microscopic changes in AD brain

Relying on gross visual changes in the brain is often a misleading means to characterize AD. Not only are these changes seen in several other types of dementia, they can also be observed in cognitively normal elderly individuals. It is only upon the histological examination of a brain specimen and the identification of two key morphological abnormalities that AD fully reveals itself: neurofibrillary tangles (NFT) and senile plaques (SP).

1.1.5.1 Intracellular neurofibrillary tangles (NFT)

In his histological examination of Auguste Deter's brain, Alois Alzheimer observed the presence of several abnormal microscopic fibrous lesions within the perikaryal cytoplasm of large pyramidal neurons (Figure 1-5A). He described them as such: "In the

center of an otherwise almost normal cell there stands out one or several fibrils due to their characteristic thickness and peculiar impregnability" (Alzheimer, 1906). These abnormal inclusions are referred to today as neurofibrillary tangles (NFT), and are considered to be one of two fundamental microscopic lesions not only associated with the disease, but required for making a definitive pathological diagnosis of AD. In fact, several studies have demonstrated a strong correlation between the extent and distribution of NFT and the severity and duration of the illness (Arriagada et al., 1992; Bierer et al., 1995). However, NFT lesions are not exclusive to AD, as they can be encountered in numerous other neurodegenerative diseases, termed tauopathies, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal lobar degeneration (FTLD), and post-traumatic type dementias, as well as normal aging (Wisniewski et al., 1979).

As Alzheimer observed, NFT are located primarily in large pyramidal neurons of Ammon's horn in the hippocampus and in the cerebral neocortex, although they can also be found in deeper structures, such as the midbrain and the hypothalamus in advanced cases (Braak et al., 1993). X-ray diffraction analysis of isolated NFT reveals a β-sheet like structural configuration (Kirschner et al., 1986), responsible for their argyrophilic and congophilic properties, and allowing them to be visualized by a variety of silver staining techniques, such as the modified Bielschowski (Figure 1-5B) or the Gallyas technique, or by fluorochrome dyes such as thioflavin S.

Figure 1-5. Neurofibrillary tangles (NFT) in AD

(**A**) Original hand drawing by Alois Alzheimer of NFT from the brain of Auguste Deter. (adapted from Maurer et al., 1997) (**B**) Photomicrograph of the hippocampus of a patient with AD (modified Bielschowsky silver stain; original magnification, 640x). Neurofibrillary tangles appear brown and have a distinct fibrillar morphology. The neuron at the top (right of center) shows granulovacuolar degeneration with characteristic argyrophilic core and surrounding clear halo. The major component of tangles are paired helical filaments containing polymerized hyperphosphorylated microtubule-associated protein Tau. (Image courtesy of Jean-Paul Vonsattel; Columbia University, NY)

Over the past several decades, much has been learned about the morphological features of NFTs. They are composed of several paired helical filaments (Kidd, 1963), comprised themselves of two axially opposed helical filaments measuring 10 nm in diameter. These are wound around each other in a helical configuration with a regular periodicity of 80 nm (Kidd, 1964; Terry et al., 1964; Wisniewski et al., 1976) --hence the name, paired helical filaments (PHF). The exact morphological features of each individual filament is not fully understood, but are thought to consist of two or more protofilaments (reviewed by Castellani et al., 2010). In terms of their biochemical constitution, it is widely accepted that the major proteinaceous component of NFT is the microtubule-associated protein tau (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). The tau protein found in NFT has been shown to be abnormally hyper-phosphorylated at several very specific sites, which may enhance its propensity

to aggregate (Garver et al., 1996; Lee et al., 1991). In addition to the tau protein, a wide variety of molecules have been shown to be associated with NFT (reviewed by Castellani et al., 2010; Smith, 1998). These include, but are not limited to: cytoskeletal proteins (e.g. vimentin and tropomyosin), protease-related elements (e.g. ubiquitin, α1-antichymotrypsin and α1-antitrypsin, cathepsins B and D), proteoglycans (e.g. heparan and chondroitin), inflammatory proteins such as cytokines and complement molecules (reviewed by Kalaria, 1993), and even the β-amyloid protein (Hyman et al., 1989), which will be discussed later.

1.1.5.2   Senile plaques (SP)

In addition to NFT, Alois Alzheimer noted the presence of another unusual lesion in Auguste Deter's brain, which he described as "numerous small miliary foci" made up by "the storage of a peculiar material in the cortex" (Alzheimer, 1906). What Alzheimer described are the second characteristic lesions found in the brains of AD patients, senile plaques (SP), also known as neuritic plaques. Of note, the association between dementia in the elderly and SP was not a novel observation by Alzheimer. "Miliary foci" lesions had already been reported prior to Alzheimer, by Blocq and Marinesco, who are credited as having provided the first description of SP in an elderly epileptic patient (reviewed by Buda et al., 2009), and later by Beljahow and Redlich and later confirmed by Leri in association with senile dementia (reviewed by Castellani et al., 2008). However, the combination of both NFT and SP, as well as the early age of onset (51 years old) gave a strong indication that Auguste Deter's dementia was perhaps a novel disease modality.

SP are complex extracellular sphere-like lesions ranging between 10 and 200 μM in diameter. They were originally identified and characterized through the use of the Bielschowski silver technique (Figure 1-6), due to the presence of a dense core accumulation of an "amyloid substance" which was confirmed by Divry in 1927 with Congo red staining (reviewed by Boller et al., 2007). However, the proteinaceous content of SP was not known for another 50 years, until Glenner and colleagues isolated and described the sequence of the peptide found in SP (Glenner et al., 1971a; Glenner et al., 1971b), which was found to be a 4-KD peptide with a β-sheet configuration, termed β-A4 (Beyreuther et al., 1986; Glenner and Wong, 1984; Masters et al., 1985a; Masters et al., 1985b; Wong et al., 1985), and later found to be a metabolic product of the amyloid precursor protein, APP (Kang et al., 1987), as we will discussed below.

Through the use of immunohistochemical techniques with antisera, we know today that several forms of SP can be encountered within the brains of elderly individuals, distributed predominantly within the cerebral cortex (Figure 1-6A) and hippocampus and within the grey matter of the neocortex. However, they can be classified into two main types, depending on their extent and severity (reviewed by Castellani et al., 2010; Perl, 2010). The classical senile or neuritic plaques have a dense core of β-A4 peptide and vary in size, between 10 and 50 μM in diameter. They are usually arranged in a radial fashion and are often surrounded by a corona of abnormally thickened neuronal processes (dystrophic neurites), which also stain with silver impregnation (Figure 1-6B, C). Often one or more microglial cells can be found in the periphery of SP, but whether or not they are merely reacting to the presence of SP or involved directly in a neuroinflammatory pathogenic cascade remains unclear (reviewed by Krause and Muller, 2010). Dense core senile plaques can also occur in the absence of any accompanying dystrophic neurites. It is often speculated that these types of plaques

are the remnants of previous neuritic plaques (Wisniewski et al., 1982), and are sometimes referred to as burned-out plaques or end-stage plaques. However, due to the cross-sectional nature of these observations, these interpretations remain speculative at best. The second type of plaques is made up of diffuse focal deposits of β-A4 in the cerebral cortex and occur in the absence of dystrophic neurites (Yamaguchi et al., 1989). Diffuse plaques tend to be more heterogeneous than neuritic plaques, with an average diameter of 20 µm, with larger plaques being far less common than their smaller counterparts (reviewed by Castellani et al., 2010). These plaques also stain with silver-based stains and are commonly found in the brains of cognitively normal elderly individuals, which raises the question as to whether or not they are a result of normal aging or indicative of an abnormal process. Nevertheless, they represent the earliest cerebral lesions found in AD and persist even in advanced AD cases, even making up the majority of β-A4-immunoreactive material found in these brains (Ikeda et al., 1989). It is not clear whether diffuse plaques evolve over time into neuritic plaques or whether they represent kinetically and thermodynamically different and distinct mechanisms.

Similar to NFT, amyloid deposits and SP are not exclusive to AD. In fact, several studies have failed to show a significant correlation between the extent of plaque pathology and the severity of the disease (Terry et al., 1991; Wisniewski et al., 1989). In addition, amyloid deposits can be found in several other conditions, including Down syndrome, dementia pugilistica, diffuse Lewy body disease and in acute traumatic brain injuries (Mann and Jones, 1990), making it difficult to establish how amyloid deposits contribute to AD or even if they are the major causative agent of neuronal degeneration.
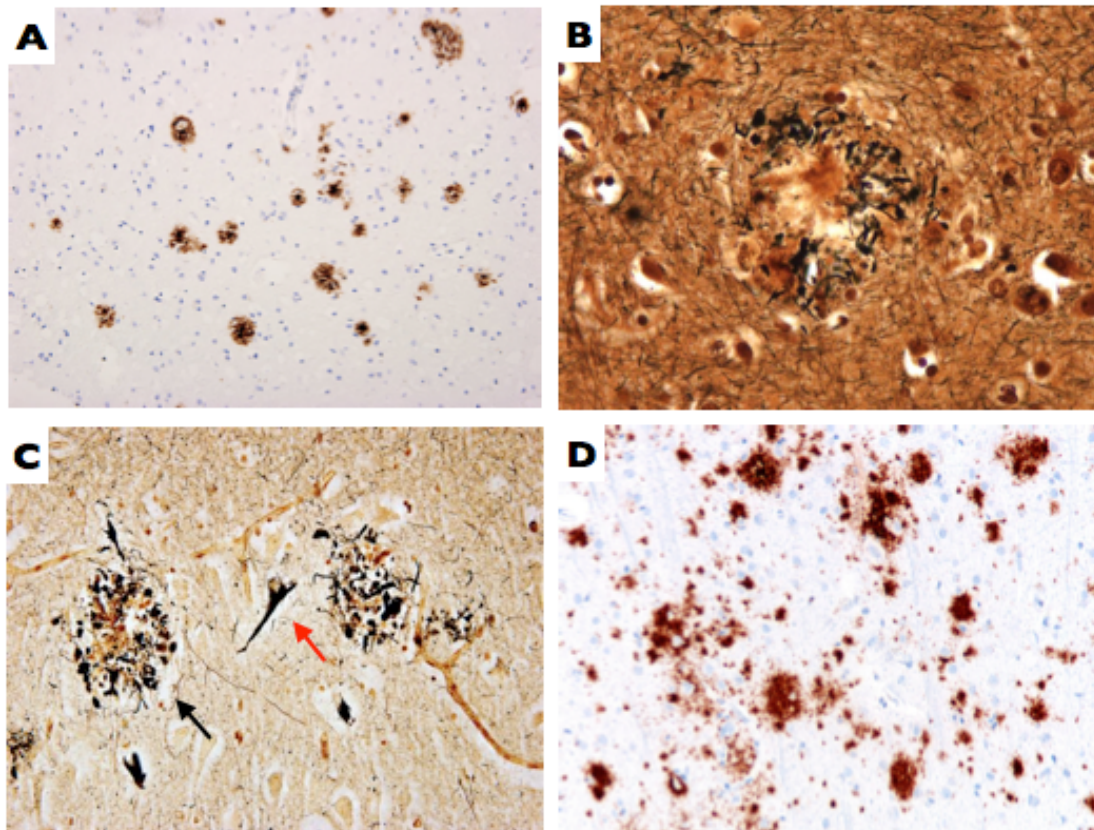
Figure 1-6. Different types of senile plaques in AD

(**A**) Temporal cortex of a patient with Alzheimer's disease (immunohistochemical stain; original magnification, 100×): the microscopic appearance of an immunohistochemical preparation using an antibody directed against the components of beta- amyloid (4G8; a gift from Dr. Robakis). This antibody selectively decorates the numerous senile plaques present in this case of advanced Alzheimer's disease and demonstrates the extent of amyloid accumulation that one may encounter in the terminal phases of the disease. (**B**) Senile plaque with neuritic components (Cerebral cortex; modified Bielschowsky silver stain). Many silver-positive (black), abnormally thickened,neuronal processes (dystrophic neurites) are arranged radially at the periphery of the senile plaque. The central amyloid core is brown and immediately surrounded by a clear zone. (Image courtesy of Jeau-Paul Vonsattel; Columbia University, NY). (**C**) Photomicrograph of the temporal cortex of a patient with Alzheimer's disease (modified Bielschowski stain; original magnification, 400×). Two senile (neuritic) plaques with a neurofibrillary tangle between them are shown.

1.1.6 Synaptic dysfunction in AD

Because of their prominence in AD, it was long speculated that NFT and SP played a causative role in the disease. Indeed, early studies hinted at a possible correlation between the severity of dementia and SP (Blessed et al., 1968) and NFT (Wilcock and Esiri, 1982). However, several subsequent studies have since failed to corroborate these findings and it is widely accepted today that there is only a weak correlation between the extent of plaque lesions and the severity of dementia. Alternatively, it appears that synapse loss and synaptic dysfunction may be more directly related to the severity of dementia than the lesions themselves (Delaere et al., 1989; Terry et al., 1991).

Ultrastructural and immunohistochemical studies from Masliah (Masliah et al., 1993; Masliah and Terry, 1993; Masliah et al., 1989; Terry et al., 1991) and Scheff (DeKosky et al., 1996; Scheff and Price, 1993, 1998; Scheff et al., 2001) have demonstrated a marked loss of synaptic elements, such as synaptophysin, SV2 and p65 in the brains of AD patients. They concluded that as much as 45% of synaptic boutons are lost in AD patients compared to normal controls, especially in the neocortex and hippocampus. They postulated that the loss of these critical elements, responsible for neuronal communication, constitute the principal morphological changes directly linked with cognitive impairment in AD. The exact mechanism(s) by which these changes occur have not been fully elucidated. However, there is ample evidence to suggest that the soluble forms of the β-amyloid peptide interferes directly with synaptic function (reviewed by Nimmrich and Ebert, 2009), leading to degeneration of the synapses and retraction of dendritic spines (discussed later).

1.1.7   Diagnosis of AD

Since Alzheimer's original description of the disease, post-mortem brain examinations still remain the most accurate and definitive means by which AD is diagnosed. However, a probable diagnosis of AD upon neurologic examination can be made in most cases, especially if other potential underlying causes of the symptoms have been ruled out. The absence of reliable fluid-based biomarkers for AD have deflected the focus towards various brain imaging techniques, which can help visualize and identify changes in the structures in the brain that are associated with memory (Table 1-2). For example, computed tomography (CT) scans and magnetic resonance imaging (MRI) have been used as tools to visualize and measure brain atrophy in AD patients (Fox and Freeborough, 1997; Fox et al., 1996). Unfortunately, the specificity of these findings are sometime questionable since other conditions can also lead to atrophy of the brain. In addition, normal aging has also been shown to be associated with a certain degree of atrophy of the brain (reviewed by Scheltens and Korf, 2000).

Alternatively, imaging studies designed to address brain function and metabolism are revealing themselves to perhaps be better alternatives for diagnostic purposes. Functional magnetic resonance imaging (fMRI), positron emission tomography (PET) scans and single photon emission computed tomography (SPECT) have all been used to measure blood flow and glucose metabolism in the brain, which can be valuable in identifying potential functional abnormalities as early as the mild cognitive impairment (MCI) stages of AD (reviewed by Craig-Schapiro et al., 2009). More recently, the development and testing of various amyloid PET ligands have yielded promising results in the past several years. For example, one such agent, ^{11}C-labelled Pittsburg compound B, or PIB has been shown to have an increased retention in the frontal, parietal, temporal, and occipital cortices and striatum in AD, leading to nearly all such

patients to test PIB-positive (Klunk et al., 2004; Pike et al., 2007; Rabinovici et al., 2007; Rowe et al., 2007). The goal of all of these techniques is to hopefully provide a suitable method not only for confirming an AD diagnosis in symptomatic cases but perhaps to pinpoint those individuals in the pre-clinical stages of the disease.

Select Candidate Fluid and Imaging Biomarkers of AD

Fluid-based markers

- Cerebral spinal fluid (CSF)
    - $A\beta 42$: decreased in AD; decreased in subjects with brain amyloid deposition; predictive of conversion from MCI to AD
    - $A\beta 40$: no difference in AD
    - $A\beta 40/42$ ration: discriminates AD from normal; predictive of conversion from MCI to AD
    - Tau/pTau: increased in AD; pTau[231] predicts conversion from MCI to AD
    - Tau/$A\beta 42$ ratio: increased in AD; predictive of conversion from normal to MCI to AD
    - Isoprostanes: increased in postmortem and antemortem AD CSF; predictive of conversion from normal to MCI to AD; increased in preclinical FAD mutation carriers
    - $\alpha 1$-antichymotrypsin: mixed results
    - Interleukin-6: mixed results
    - various inflammation markers: mixed results

- Plasma/serum
    - $A\beta 42$: mixed results; increased in FAD
    - $A\beta 40$: mixed results; decreased in FAD
    - Isoprostanes: mixed results
    - $\alpha 1$-antichymotrypsin: mixed results; predictive of AD risk
    - Interleukin-6: mixed results

- Urine
    - Isoprostanes: mixed results

Imaging modalities

- CT and MRI: regional atrophy in AD; whole brain atrophy in AD; predictive of conversion from MCI to AD; predictive of conversion from normal to MCI

- fMRI: altered activation in AD; altered activation in MCI

- FDG-PET: regional hypometabolism in AD; predictive of conversion from MCI to AD

- $H_2{}^{15}O$-PET: altered activation in AD

- SPECT: altered regional cerebral perfusion in AD; predictive of conversion from MCI to AD

- ASL-MRI/contrast-based MRI: regional hypoperfusion in AD

- FDDNP-PET: increased retention in AD and MCI brain

- PIB-PET: increased retention on AD brain; increased retention in a subset of cognitively normal controls; detects cerebral amyloid angiopathy

- Other PET amyloid imaging agents: increased retention in AD brain

- PET markers of microglial activation: increased retention in AD and MCI brain

Table 1-2. Select candidate fluid and imaging biomarkers of AD

(adapted from Craig-Schapiro et al., 2009)

1.2   The amyloid precursor protein (APP) family of genes

Over the years, there has been tremendous progress towards understanding the molecular pathways and signaling changes that occur in the pathology of AD. Unfortunately, the event(s) that trigger and alter these molecular pathways have remained, to a large extent, poorly understood. The discovery of the β-A4 peptide as the main constituent of amyloid plaques (Beyreuther et al., 1986; Masters et al., 1985a; Masters et al., 1985b; Wong et al., 1985) was an undeniably important leap forward in AD research. However, up to that point the provenance of the peptide remained a mystery. Two years later, its source would finally be revealed when the amyloid precursor protein (APP) was cloned for the first time (Kang et al., 1987).

1.2.1   Overview

Since it was originally cloned, the amyloid precursor protein, APP (Kang et al., 1987) has been found to be an evolutionary highly conserved gene family which, in addition to APP, includes the two homologous mammalian genes encoding the proteins APP-like protein-1, APLP1 (Wasco et al., 1992) and APP-like protein-2, APLP2 (Wasco et al., 1993b), as well as the *Drosophila melanogaster* homologue, APPL (Rosen et al., 1989) and the *Caenorhabditis elegans* homologue APL-1 (Daigle and Li, 1993). The APP family proteins are intriguing due to their sequence and structure, which have lead to many proposed, but unclear, functions. However, it is becoming increasingly clear, as we'll discuss in more detail below, that the APP gene family proteins have, at the very least, partly overlapping, if not redundant important functions.

Of all the members of the gene family, APP has been the most well studied and characterized protein due to its role in AD. Much of what we will discuss below applies to APP; additional details will be provided for the other members when necessary.

1.2.2. Genetics

In humans, the gene for APP is located on chromosome 21 at 21q21.2 (Korenberg et al., 1989; Patterson et al., 1988; Robakis et al., 1987; Tanzi et al., 1987) and contains 19 exons (Yoshikai et al., 1991), while APLP1 and APLP2 are found on chromosome 19 (Wasco et al., 1993a) and chromosome 11 (von der Kammer et al., 1994) respectively. At least 8 different isoforms of APP can be generated by alternative splicing of exons 7, 8 and 15 (Figure 1-7), all coding for part of the ectoplasmic portion of the molecule (reviewed by Menendez-Gonzalez et al., 2005). Although APP is ubiquitously expressed, some isoforms are preferentially expressed in different cell types. In non-neuronal cells, the longer isoforms containing exon 7 (APP^{751}) or exons 7 and 8 (APP^{770}) predominate, while neurons express primarily the shorter isoform lacking exons 7 and 8 (APP^{695}) (Kang and Muller-Hill, 1990). APP isoforms devoid of exon 15 are typically not expressed in neurons, but can be found in many other cell types, where they function as the core protein for a proteoglycan called appican (Pangalos et al., 1995).

As for the other family members, APLP2 contains three exons that can be alternatively spliced in a manner very similar to APP (Sandbrink et al., 1994), while the APLP1 gene only produces a single transcript (Lenkkeri et al., 1998), as is the case for APPL and APL-1 (Daigle and Li, 1993). As for their expression profiles, APLP2 is ubiquitously expressed (Wasco et al., 1993b), but APLP1 is restricted to the nervous system (Lorent et al., 1995; Slunt et al., 1994). In *C. elegans*, APL-1 is expressed in multiple tissues

(Hornsten et al., 2007), whereas the Drosophila APPL protein is exclusively neuronal (Luo et al., 1990).

Figure 1-7. Exon-intron organization of the human APP (hAPP) gene

Boxes represent exons, colored boxes represent isoform-specific exons as shown. Horizontal lines indicate introns. The whole gene is approximately 400 kb in length (elements are not drawn to scale).
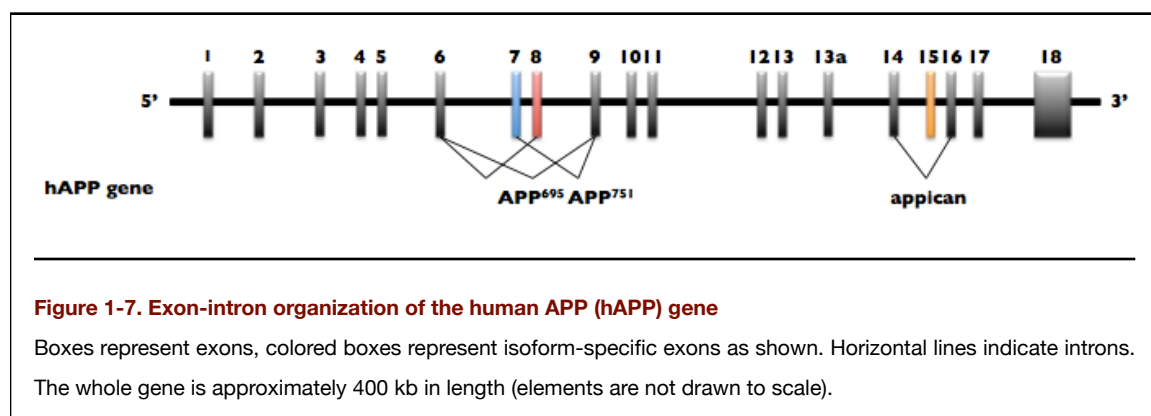
The overall extensive similarities in the amino acid sequences of APP and APLPs and their overlapping brain expression patterns strongly suggest that these proteins fulfill highly redundant biological roles in mammals.

1.2.3 Protein structure

The APP family member proteins are all type I integral membrane proteins, which share the same basic domain structures (reviewed by Gralle and Ferreira, 2007): a single membrane-spanning domain, a large ectoplasmic N-terminal region and a shorter cytoplasmic C-terminal region. The ectoplasmic region of APP, which constitutes the major part of the protein is comprised of two distinct, largely independently-folding structural domains, E1 and E2 (Figure 1-8A). The former can be further subdivided into several sub-domains (Figure 1-8A, B), including a heparin-binding/growth factor-like domain (HBD/GFLD), a copper-binding domain (CuBD) and a zinc-binding domain (ZnBD). Similarly, the E2 domain contains an additional HBD/GFLD, as well as a random coil (RC) region. As mentioned above, the splicing of exon 15 in APP^{770} creates a chondroitin sulfate glycosaminoglycan (GS GAG) attachment site used in astrocytes but

not in neurons (Pangalos et al., 1995). The E1 and E2 domains are linked by an acidic region, rich in aspartic and glutamic acid residues and a Kunitz-type protease inhibitor domain (KPI) --only present in the longer isoforms APP^{751} and APP^{770}-- thought to function in regulating cerebral thrombosis (Xu et al., 2005). The KPI domain is immediately followed by a seemingly random short stretch of 19 amino acids without significant homology to other known proteins and without a recognizable structure (Richards et al., 1995). This is followed by the transmembrane domain (TM), which includes part of the β-amyloid peptide (Aβ) sequence (Figure 1-8C; discussed below). Lastly, the cytoplasmic tail (IC) of APP contains a protein interaction motif, namely the YENPTY sequence (Lai et al., 1995) (including the NPXY internalization signal), conserved in all APP homologues.

Classical N- and O-glycosylation (Weidemann et al., 1989), as well as sulfation and phosphorylation of several tyrosine residues of APP occur on both sides of the membrane (Figure 1-6B) during trafficking through the endoplasmic reticulum (ER) and the Golgi apparatus (reviewed by Turner et al., 2003), increasing the structural complexity of APP and APLPs. While no clear role for APP glycosylation could be demonstrated in chinese-hamster ovary (CHO) cells (Pahlsson and Spitalnik, 1996), N- and O-glycosylation of the extracellular portion of APP has been suggested to be necessary for phosphorylation of a threonine residue (Thr^{668}) in its cytoplasmic domain during neuronal differentiation (Ando et al., 1999).
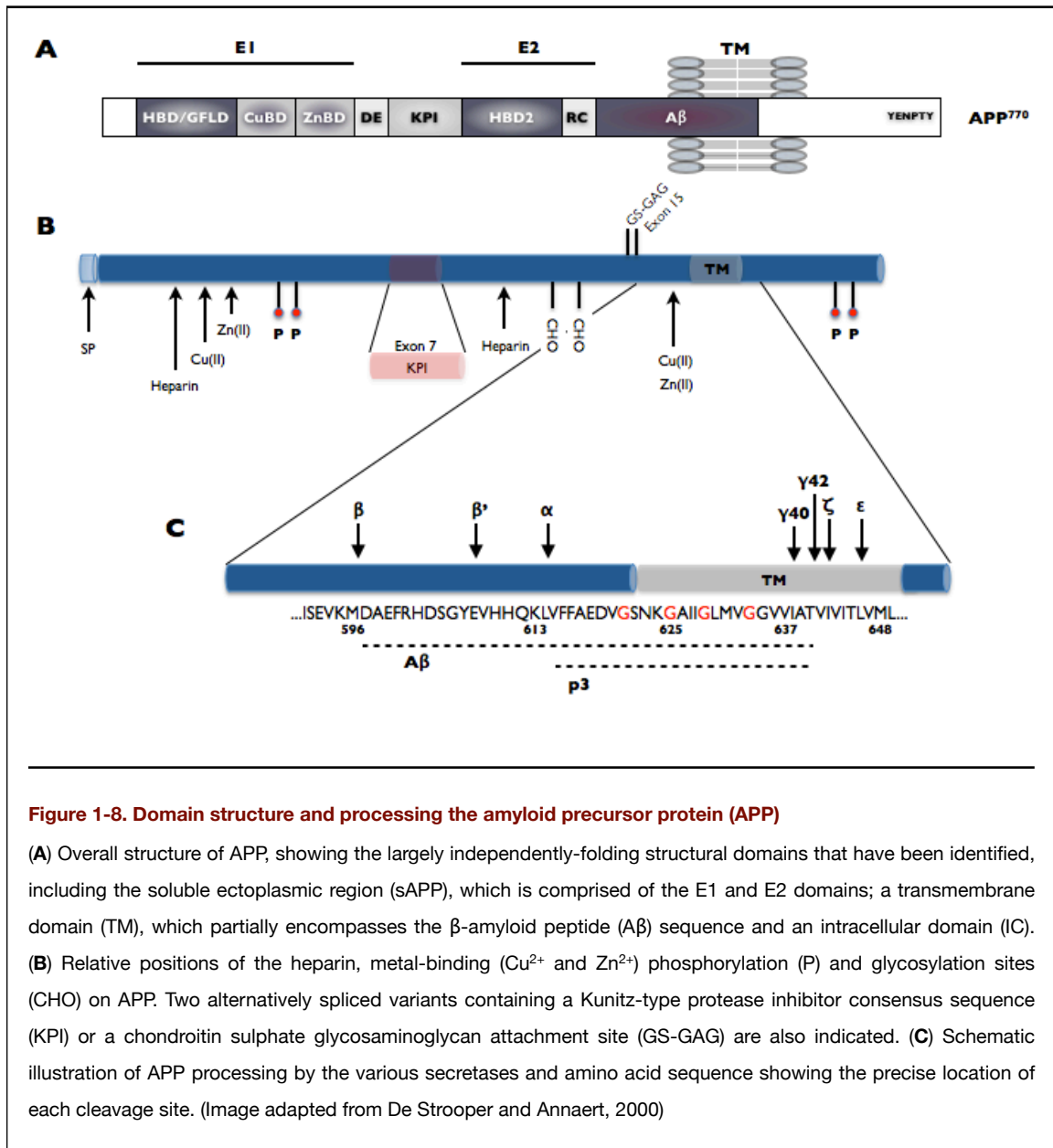
Figure 1-8. Domain structure and processing the amyloid precursor protein (APP)

(**A**) Overall structure of APP, showing the largely independently-folding structural domains that have been identified, including the soluble ectoplasmic region (sAPP), which is comprised of the E1 and E2 domains; a transmembrane domain (TM), which partially encompasses the β-amyloid peptide (Aβ) sequence and an intracellular domain (IC). (**B**) Relative positions of the heparin, metal-binding (Cu^{2+} and Zn^{2+}) phosphorylation (P) and glycosylation sites (CHO) on APP. Two alternatively spliced variants containing a Kunitz-type protease inhibitor consensus sequence (KPI) or a chondroitin sulphate glycosaminoglycan attachment site (GS-GAG) are also indicated. (**C**) Schematic illustration of APP processing by the various secretases and amino acid sequence showing the precise location of each cleavage site. (Image adapted from De Strooper and Annaert, 2000)

1.3   *Proteolytic processing of APP and formation of the β-amyloid peptide (Aβ)*

In addition to the many post-translational modifications outlined above, APP is further modified through a series of cleavage processes, generating a wide variety of fragments including the Aβ peptide (reviewed by Walsh et al., 2007). Two related proteolytic

pathways mediated by three distinct cleavage events have been identified.

1.3.1 α-Secretase and the non-amyloidogenic pathway

The first pathway, originally described by Esch *et al.* (Esch et al., 1990), is initiated by a protease referred to as α-secretase, which cleaves APP between lysine[612] and leucine[613] (APP[695] numbering; Figure 1-8C), producing two fragments, the N-terminal ectodomain sAPPα and an 83-amino-acid-long C-terminal membrane-bound fragment C83 (Figure 1-9A). Since this cleavage occurs within the sequence of the Aβ domain itself (residues 16 and 17 of Aβ), this pathway is commonly referred to as the non-amyloidogenic pathway for it precludes generation of the Aβ peptide.

Although this pathway has been known for some time, the complete picture of the processing events and the enzymes involved have not been fully unraveled. α-Secretase candidates have been identified and characterized as being all zinc metalloproteinases, members of the ADAM (a disintegrin and metalloproteinase) family of proteases (reviewed by De Strooper and Annaert, 2000). These include TACE (tumor necrosis factor-α converting enzyme), also know as ADAM17 (Black et al., 1997; Moss et al., 1997), as well as ADAM10 (Lammich et al., 1999) and MDC-9, also known as meltrin-γ or ADAM9 (Koike et al., 1999).

Initially, it was thought that this cleavage may be physiological, thereby preventing the production of Aβ in AD (Esch et al., 1990; Sisodia et al., 1990). However, this view may be an oversimplification. Cleavage of APP by α-secretase occurs at the cell surface (Parvathy et al., 1999) and only a small percentage of APP (compared to the total cellular pool) is present at the cell surface (Kuentzel et al., 1993; Sambamurti et al.,

1992) due to the rapid removal of cell-surface APP (Koo et al., 1996). Hence, only a small fraction of the total pool of APP is cleaved by α-secretase in most cell types, leaving most of APP available for the alternative amyloidogenic pathway, which we will outline below.

Figure 1-9. Proteolytic processing of APP

(**A**) APP undergoes two distinct cleavages within its juxtamembrane region. The predominant cleavage is mediated by α-secretase and results in the secretion of sAPPα and the generation of an 83-amino-acid-long membrane-tethered fragment. The alternative, amyloidogenic, cleavage occurs just 16 residues N-terminal to the α-site and is brought about by BACE. β-Cleavage results in the secretion of sAPPβ and the production of the C99 fragment. C99 and C83 undergo heterogeneous cleavage by γ-secretase leading to the secretion of Aβ and p3 and the release of ICD into the cytoplasm. (**B**) Aβ$_{42}$ is prone to self-aggregate to form soluble globulomers consisting of 2-14 Aβ monomers, Staining of Aβ42 oligomers with an Aβ42 globulomer-specific antibody in a cortical section of a patient with AD (Barghorn et al., 2005).

1.3.2   β-Secretase and the amyloidogenic pathway

The second known proteolytic pathway leads to the production of Aβ, and is typically referred to as the amyloidogenic pathway. It is initiated by the protease β-secretase, which was identified through four independent approaches (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), as BACE1 (β-site APP-cleaving

enzyme). Cleavage of APP by BACE1 (De Strooper and Annaert, 2000) occurs between methionine[596] and aspartate[597] of APP[695] (Figure 1-8C), producing two fragments, the secreted N-terminal ectodomain sAPPβ and a remaining 99-amino-acid-long fragment C99 (Figure 1-9A), encompassing the Aβ peptide. Additionally, BACE1 can cleave APP at another site, between tyrosine[606] and glutamate[607] (Figure 1-8C), releasing a longer sAPPβ and an 89-amino-acid-long fragment C89. This results in a shorter form of the Aβ peptide previously detected in plaques (Masters et al., 1985b; Naslund et al., 1994) and in culture (Haass et al., 1992; Simons et al., 1996).

BACE1 is a type I integral membrane protein with a putative pro-domain and two DT/SGT/S motifs on its extracellular domain (Hussain et al., 1999; Vassar et al., 1999), a classical signature of the catalytic domain of aspartyl proteinases. BACE1 is synthesized as a proenzyme, whose pro-domain is cleaved by furin-like protease as it is trafficked to the plasma membrane through the secretory pathway (reviewed by Tang, 2009). BACE1 then cycles to the cell surface and endosomes, and at steady-state is found primarily in the late Golgi-TGN compartments and endosomes (Huse et al., 2000). Additionally, the C-terminus of BACE1 contains an acidic dileucine motif ([495]DDISLLK[501]) that targets BACE1 from the plasma membrane to endosomes (Huse et al., 2000). Phosphorylation at serine[498] has been implicated in its trafficking between early endosomes and the TGN/late endosomes (Walter et al., 2001). The optimal pH of BACE activity is approximately 4.5, suggesting that the β-site cleavage of APP occurs preferentially in more acidic compartments, such as in endosomes (Vassar et al., 1999). It has also been suggested that BACE1 cleavage can occur in lipid rafts (Ehehalt et al., 2003). At least one other human homologue of BACE1 has been identified, BACE2 (Hussain et al., 2000), which interestingly is a better α- than β-secretase (Farzan et al., 2000). Nevertheless, BACE2 is expressed at very low levels in the brain, and is mostly

restricted to glial cells (Laird et al., 2005); a potential role for BACE2 in AD has yet to be identified.

1.3.3 γ-Secretase cleavage and production of Aβ

As a result of either α-secretase or β-secretase cleavage of APP, three membrane-bound C-terminal fragments (CTFs) are produced, C99 or C89 and C83, which all serve as substrates for the final cleavage event, namely γ-secretase cleavage. γ-Secretase is a unique aspartyl protease complex (Walsh et al., 2007), which unlike α- and β-secretases, acts deep within the membrane and cleaves APP at multiple sites (Zhao et al., 2004), releasing either Aβ and intracellular C-terminal domain fragments (ICDs) or p3 and ICDs (Figure 1-9A). However, while the two predominant forms of Aβ and p3 terminate at valine^{637} (Aβ$_{40}$ and p3$_{40}$) and alanine^{639} (Aβ$_{42}$ and p3$_{42}$) (Haass et al., 1992; Figure 1-8C), isolated ICDs are shorter than expected and begin at sites 9-10 amino acid downstream of those residues (Gu et al., 2001). This inconsistency led to the recognition that γ-secretase-cleavage of CTFs occurs at three distinct sites. The first, referred to as the ε-site (Figure 1-8C), happens 9-10 residues downstream of Valine^{637} and produces a 49-50 amino-acid fragment C49/C50 (Gu et al., 2001; Weidemann et al., 2002). The second cleavage occurs 6 residues downstream of Valine^{637} and is referred to as the ζ-site (Kakuda et al., 2006; Zhao et al., 2004). Finally, the last cut occurs at the γ-site to give rise to Aβ$_{40}$/Aβ$_{42}$ and p3$_{40}$/p3$_{42}$. In all, several Aβ species are generated and have been isolated, including Aβ$_{40}$, Aβ$_{42}$, Aβ$_{43}$, Aβ$_{45}$, Aβ$_{48}$ and Aβ$_{49}$, and only two species of ICDs, C49 and C50 (Kakuda et al., 2006).

Unlike its counterparts, the γ-secretase enzyme is a protein complex (Figure 1-10) made up of at least four individual components (reviewed by Wolfe, 2008): aph-1

(anterior pharyx defective-1), nicastrin, presenilin-1 (PS1) or presenilin-2 (PS2) and pen-2 (presenilin enhancer-2) (Edbauer et al., 2003; Francis et al., 2002; Goutte et al., 2000; Yu et al., 2000). In humans, aph-1 exists as two isoforms, aph-1a and b --mice have a third isoform, aph-1c. It is thought to be a scaffolding protein that functions to stabilize the complex and contributes to its trafficking (Niimura et al., 2005; Takasugi et al., 2003). Nicastrin has recently been demonstrated to play a critical role in substrate recognition through its ectodomain, which binds the N-terminus of membrane-bound substrates (Shah et al., 2005). Upon assembly, it is believed that aph-1 and nicastrin first form a sub-complex (Hu and Fortini, 2003; LaVoie et al., 2003; Morais et al., 2003; Shirotani et al., 2004) to which PS1 or PS2 protein subsequently binds.

PS1 and PS2 are nine-transmembrane domain proteins that are thought to possess the actual protease activity (Yu et al., 1998). Upon activation, full-length presenilin protein is cleaved in the cytoplasmic loop between the 6th and 7th transmembrane region to generate stable N- and C-terminal fragments (Thinakaran et al., 1996a). These fragments stay in close proximity in the membrane to generate the active form of the protein complex. It is this active form that then joins the aph-1/nicastrin sub-complex, before pen-2 presumably comes in to complete and perhaps to further stabilize the complex or to facilitate auto-cleavage of PS (LaVoie et al., 2003; Niimura et al., 2005).

The sub-cellular localization of γ-secretase and its activity remain a matter of controversy, as each subunit of the enzyme has been found in multiple organelle including the endoplasmic reticulum (ER), ER-Golgi intermediate compartments, Golgi apparatus, endosomes, lysosomes, phagosomes, plasma membrane, and even mitochondria (reviews by Marks and Berg, 2010; Tang, 2009).

Figure 1-10. The γ-secretase complex

Aph-1 and nicastrin form a stable sub-complex early during assembly, followed by entry of the presenilin holoprotein to form a trimeric intermediate complex (Aph-1::immature nicastrin::presenilin holoprotein). Maturation of nicastrin in the Golgi/trans Golgi network and entry of Pen-2 into the maturing complex promote presenilin endoproteolysis and formation of active -secretase. The active complex contains cleaved presenilin NTF and CTF as well as associated cofactors. It is currently uncertain where some assembly steps occur, what is the precise composition of the complex at each stage, what are the specific interactions among various components at each stage, and whether alternative complexes with different subunit compositions might exist.

1.4   Intracellular trafficking of APP

1.4.1   Intracellular itinerary

The pathways of APP trafficking (reviewed by Thinakaran and Koo, 2008) are depicted in Figure 1-11 (Vetrivel and Thinakaran, 2010). Nascent APP molecules mature through the constitutive secretory pathway. As it transits from the ER through the Golgi to the plasma membrane, APP is post-translationally modified (reviewed in section 1.2.3). Interestingly, as was mentioned above, only a small fraction of nascent APP molecules is present at the plasma membrane (Kuentzel et al., 1993; Sambamurti et al., 1992), estimated to be as low as ~10%, based on APP over-expression studies in cultured cells. At steady state, the majority of APP localizes to the Golgi apparatus and trans-Golgi network (TGN). In non-neuronal cells, once APP reaches the plasma membrane, it

is rapidly internalized (Koo et al., 1996; Sisodia, 1992) due to the presence of the YENPTY internalization sequence near the C-terminus of APP (residues 682-687 of APP[695]). Following endocytosis, APP is delivered to endosomes, where it can be trafficked through the endocytic and recycling compartments back to the cell surface or degraded through the lysosomal pathway.

Several adaptor proteins have been shown to bind the NPTY sequence, regulating the trafficking of APP. These include X11/mint (Miller et al., 2006), Fe65 (Borg et al., 1996), Dab1 (Homayouni et al., 1999), JIP family of proteins (Matsuda et al., 2001) and Sortin nexin 17 (Lee et al., 2008a). In addition to cytosolic adaptors, several transmembrane proteins of the low-density lipoprotein receptor-related (LDLR) family members, including LRP1, LRP1B and SorLA, have been shown to interact with APP and modulate its trafficking and processing (Andersen et al., 2005; Cam et al., 2004; Cam et al., 2005). Moreover, in neurons APP is transported through the axon *via* the fast anterograde machinery (Koo et al., 1990), accounting for the observed synaptically released Aβ pool (Lazarov et al., 2002).

1.4.2 Endocytic sorting of APP and Aβ production

The processing of APP to Aβ is profoundly modulated by its sub-cellular localization (Figure 1-11), which affects not only the efficiency but also the choice of metabolic pathway --i.e. amyloidogenic versus non-amyloidogenic pathway (reviewed by Vetrivel and Thinakaran, 2010). Much of what has been learned about this topic has originated from studies in cultured mostly non-neuronal cell lines, such as chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293) cells. Based on several of these studies, it is believed that Aβ is principally generated in the TGN and endosomes as APP is trafficked through both the secretory and recycling pathways (Ghribi, 2006;

Greenfield et al., 1999; Xu et al., 1997). This concurs with the fact that BACE1 and γ-secretase are primarily localized to these organelles (Baulac et al., 2003; Vetrivel et al., 2004) and with the fact that BACE1 is optimally active at pH 4.5, as mentioned earlier (Vassar et al., 1999). Indeed, endocytosis of APP has been found to be critical for Aβ production both in cultured cells and *in vivo* (Cirrito et al., 2008; Koo and Squazzo, 1994). Interestingly, APP bearing mutations associated with familial early-onset AD (discussed later) in a swedish kindred (APP K670N/M671L; APPswe) is more readily cleaved by BACE1 in the secretory pathway, as early as during trafficking through the Golgi (Thinakaran et al., 1996b).
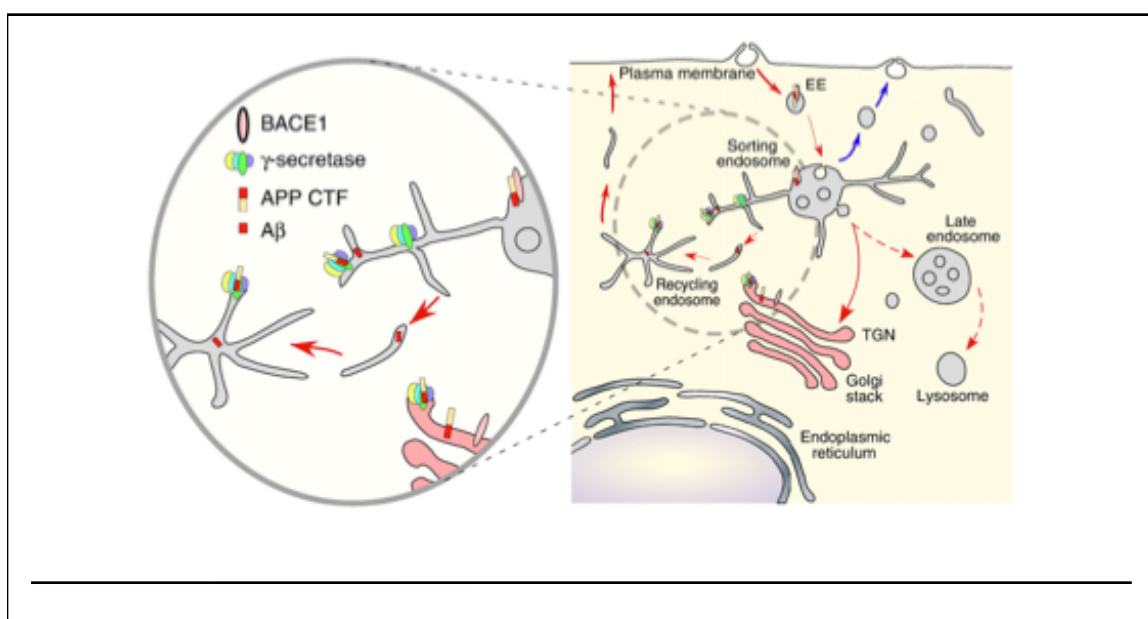
Figure 1-11. Schematic illustration of the intracellular itinerary of APP

Schematic illustration of intracellular itinerary of amyloid precursor protein (APP). Synthetic APP is trafficked through the constitutive secretory pathway to the plasma membrane (blue arrows). From the cell surface, a fraction of APP is internalized and trafficked through endocytic and recycling compartments (red arrows) to reach the cell surface, the TGN, or sorted to the lysosomes for degradation (dotted red arrows). Non-amyloidogenic processing occurs mainly at the cell surface, where α-secretase activity is abundant. Amyloidogenic processing likely occurs in the endocytic pathway as APP encounters BACE1 and γ-secretase in the endosomes and recycling organelles. γ-secretase subunits and APP CTFs are enriched in lipid raft microdomains isolated from these compartments (highlighted by a circle). EE, Early endosome. For simplicity, the constitutive secretory trafficking pathway is not indicated by arrows. (Image adapted from Vetrivel and Thinakaran, 2010)

1.5   Biological functions of APP

1.5.1   APP and APLPs loss of function studies

In order to better understand the normal biological functions of the APP family gene proteins, several labs have generated single APP- or APLP-knockout (KO) mice (reviewed by Anliker and Muller, 2006), which have largely supported the idea of evolutionary conserved functions. Case in point, deletion of each individual gene (e.g. $APP^{-/-}$, $APLP1^{-/-}$ or $APLP2^{-/-}$) yields viable and fertile mice with minor and benign phenotypic abnormalities, which are comparable for each of the family member. For example, APP-knockout mice exhibit a reduction in overall body weight and a reduction in grip strength (Muller et al., 1994), as well as an increased sensitivity to kainate-induced seizures (Steinbach et al., 1998). Their brains show a decrease in the weight and size of forebrain commissures, reactive gliosis (Zheng et al., 1995), and an increase in copper levels in the cerebral cortex and liver (White et al., 1999). They also exhibit mild cognitive impairments and behavioral deficits (Muller et al., 1994; Zheng et al., 1995), accompanied by impaired long-term potentiation (LTP) (Dawson et al., 1999). APLP1 knockout mice on the other hand show only an obvious reduction in body weight (Heber et al., 2000), whereas APLP2 knockout mice have no apparent phenotype or histological abnormalities but do have increased levels of copper in the cerebral cortex (White et al., 1999). The results from these studies are consistent with *in vitro* studies which have suggested that APP, APLP1 and APLP2 function to promote neurite outgrowth (Young-Pearse et al., 2008), neural cell migration and copper homeostasis (Heber et al., 2000; Herms et al., 2004; Muller et al., 1994; White et al., 1999; Zheng et al., 1995), and suggest that the relatively mild phenotype in the KO-mice stems from functional redundancy between each gene family member.

In contrast to single gene deletions, double-knockout mice APLP2$^{-/-}$/APP$^{-/-}$ and APLP2$^{-/-}$/APLP1$^{-/-}$ are perinatally (postnatal day 1, P1) lethal (von Koch et al., 1997), whereas APLP1$^{-/-}$/APP$^{-/-}$ mice are viable and fertile, but otherwise relatively normal albeit with a slight decrease in body weight (Heber et al., 2000). The unique viability of the APLP1$^{-/-}$/APP$^{-/-}$ double-knockout mice indicates that APLP2 may be the only family member with the key physiological role(s) necessary to compensate for the simultaneous loss of both of the other APP family members and provides strong genetic evidence for at least some unique biological functions for APLP2. Consistent with this idea, triple-knockout mice (APLP2$^{-/-}$/APP$^{-/-}$/APLP1$^{-/-}$) are perinatally lethal, with 100% penetrance, with the majority of mice showing severe cranial defects and cortical dysplasia resembling human type II lissencephaly and partial loss of Cajal Retzius cells (Herms et al., 2004), suggesting a role for APP family genes in neuronal migration (Young-Pearse et al., 2007).

Although the reverse genetic studies described above have confirmed an essential role for APP and its family members APLP1 and APLP2 during post-natal development, the precise molecular nature of this function has remained difficult to pinpoint. Nevertheless, the numerous functional domains that have been mapped to the extracellular and intracellular portions of APP, as well as its metabolites have yielded valuable clues.

1.5.2   Growth promoting effects

One of the earliest evidence for APP functions was obtained by assessing the growth pattern of fibroblasts in which the levels of APP were depleted by an antisense construct (Saitoh et al., 1989). These cells grew poorly, but the growth defect could be reversed by either parent cell conditioned medium or by treatment with partially purified

brain sAPP. The active region was subsequently mapped to a pentapeptide domain (RERMS; amino acids residues 403-407 of APP[695]) near the middle of the ectoplasmic region of APP (Ninomiya et al., 1993). The growth promoting properties of sAPPα have also been reported in a number of cells of epidermal origin, such as keratinocytes (Herzog et al., 2004; Siemes et al., 2006) and epidermal basal cells (Hoffmann et al., 2000).

1.5.3 Modulation of synaptic plasticity and memory

The activity of the pentapeptide domain of APP is not exclusive to fibroblasts. Several studies have demonstrated a role for APP, and especially sAPPα, in neuronal excitability and synaptic plasticity. For instance, it was reported that intracerebroventricular administration of either the pentapeptide alone or recombinant sAPP into brains of mice and rats lead to an increase in synaptic density as well as an improvement in learning and memory retention (Meziane et al., 1998; Roch et al., 1994; Taylor et al., 2008). Interestingly, in the latter study, they observed that this effect was accompanied by increased long-term potentiation (LTP) and enhanced N-methyl D-aspartate (NMDA) receptor-mediated current. However, this result is in conflict with the *in vitro* observations that sAPPα had a suppressive role on NMDA currents in cultured hippocampal neurons (Furukawa and Mattson, 1998).

Of note, while the vast majority of the literature has documented the effects of the Aβ peptide on learning and memory in a pathological context (discussed later), a recent study by Puzzo *et al.* suggests that its detrimental effects occur at high concentrations, whereas low concentrations positively modulate synaptic plasticity and memory (Puzzo et al., 2008).

1.5.4 Trophic properties

Of all of the proposed functions of APP, its trophic properties have been perhaps the most consistently and arguably the best established function (reviewed by Jacobsen and Iverfeldt, 2009), particularly through its secreted soluble sAPPα fragment. For instance, the binding of APP to heparan sulphate proteoglycans (HSPG) was reported to stimulate neurite outgrowth in cultured chick sympathetic and mouse hippocampal neurons (Small et al., 1994). In addition, APP and sAPPα were shown in several studies to regulate neurite elongation and branching, perhaps by mediating NGF-promoted neurite outgrowth (Milward et al., 1992). In agreement with these results, Perez *et al.* reported shorter axonal outgrowth in hippocampal neurons from APP knock-out mice compared to their wild-type counterparts (Perez et al., 1997). However, this effect was only transient, as 3 day-old axons were longer than wild-type ones, suggesting that APP initially stimulates neurite outgrowth, but inhibits it over time. It has been suggested that this inhibitory effect is due to the interaction of full-length APP with integrin β1 (Young-Pearse et al., 2008). By interfering with this interaction, sAPPα is thought to lift the inhibitory effects of full-length APP, thereby stimulating neurite outgrowth. In support of this hypothesis, Young-Pearse *et al.* showed in the same study that sAPPα was unable to induce neurite outgrowth in the absence of cellular APP expression. The secreted form of sAPLP1 and sAPLP2 were both shown to stimulate neurite outgrowth in a manner similar to that of APP.

1.5.5 Cell adhesion properties

The neurite-outgrowth-promoting functions of APP and sAPPα have led many to hypothesize that APP must also play a role in cell adhesion, since neuronal migration,

neurite outgrowth and synaptogenesis would all involve substrate adhesion (reviewed by Thinakaran and Koo, 2008). In line with this hypothesis, Shubert *et al.* reported that APP can modulate the adhesion of a murine pheochromocytoma cell line (PC12) to the substrata (Schubert et al., 1989). Moreover, a RHDS motif near the extralumenal portion of APP (amino acids 328-332 of APP[695]), within the Aβ region, has been identified and shown to stimulate neurite outgrowth in a variety of cell lines, by promoting cell adhesion (Ghiso et al., 1992; Jin et al., 1994). It is thought that this region acts in an integrin-like manner and can, accordingly, be blocked by RGDS peptide sequence derived form the fibronectin-binding domain. Fittingly, APP has been shown to colocalize with integrins on the surface of axons and at sites of adhesion (Storey et al., 1996; Yamazaki et al., 1997). Evidence of its interactions with several extracellular matrix proteins, such as laminin, collagen and heparan sulfate proteoglycans (HSPG) (Beher et al., 1996; Buee et al., 1993b; Narindrasorasak et al., 1992) further strengthens the notion of the adhesion promoting properties of APP.

Finally, X-ray crystallography analysis has revealed that APP and APLPs can form antiparallel homo- and hetero-dimers (discussed in further detail below) through their ectodomains (Soba et al., 2005; Wang and Ha, 2004). The former study further demonstrated that these type of interactions may be involved in cell-cell adhesion in mouse embryonic fibroblasts in an APLP2-dependent manner (Soba et al., 2005). However, a recent study reported that although all mammalian homologues can form parallel (*cis*) interactions, antiparallel (*trans*) interactions were limited to APLP1 (Kaden et al., 2008), suggesting a specific role for APLP1 in adhesion.

1.5.6 Additional functions

In addition to those functions mentioned above, several more have been reported for APP, including a role in neurogenesis. For example, Moya *et al.* showed that APP is developmentally regulated and its expression is increased at the time of synaptogenesis (Moya et al., 1994) and may play a role in the correct migration on neuronal progenitors into the cortical plate (Young-Pearse et al., 2007). APP has also been proposed to have a role in axonal outgrowth after traumatic brain injury (Leyssen et al., 2005) and a protective role in ischemic brain injuries (Smith-Swintosky et al., 1994). Finally, sAPPα has been shown to enhance long-term neuronal survival in rat cortical cell cultures (Araki et al., 1991) and to prevent death in human cortical cell cultures deprived of glucose and exposed to excitotoxins (Mattson et al., 1993), and even to Aβ-induced toxicity (Goodman and Mattson, 1994).

1.6   The etiology of Alzheimer's disease (AD)

Genetically, AD is usually classified into two forms: (1) Familial cases with Mendelian inheritance of predominantly early-onset age (<60 years-old), usually referred to as early-onset familial AD (EOFAD), and (2) ostensibly called sporadic cases, with less apparent or no familial aggregation of usually late-onset age (>65 years-old), typically called late-onset AD (LOAD). It is important to stress that this traditional categorization is overly naive, as there are rare cases of EOFAD without evidence of strong Mendelian transmission, while on the other hand, LOAD is frequently observed with a strong familial clustering, which can mirror a Mendelian pattern.

1.6.1 Early-onset familial Alzheimer's disease (EOFAD)

As mentioned above, a small subset (~1% to ~5%; Maslow, 2010) of AD patients develop clinical symptoms (reviewed in 1.1.3) before the age of 60, sometimes as early as in their 30s. Positional cloning methods have led to the identification of three distinct genetic loci --APP on chromosome 21q, PSEN1 (presenilin-1) on chromosome 14q and PSEN2 (presenilin-2) on chromosome 1-- that cause AD with extremely high penetrance in mutation carriers (reviewed by Tanzi and Bertram, 2005). To date, a total of 32 autosomal dominant mutations have been identified in APP, 181 in PSEN1 and 14 in PSEN2 (AD mutation database; reviewed in Bertram et al., 2010). The vast majority of these EOFAD mutations appear to confer one of two related biochemical phenotypes: an increased production of the Aβ peptide (Cai et al., 1993; Citron et al., 1992) or an increased ratio of cerebral Aβ42 relative to Aβ40 (Suzuki et al., 1994). While it remains unclear how these mutations alter the ratio of Aβ species generated from APP, it appears that they alter how APP is enzymatically processed at the γ-secretase site to produce Aβ (Price et al., 1998).

The identification of these mutations have lent strong support for the amyloid cascade hypothesis, first proposed by Glenner (Glenner and Wong, 1984), which postulates a central causative role for Aβ in initiating the AD pathogenic cascade and argues that the neurodegenerative process, including the development of NFT, is a consequence of an imbalance between the generation and clearance of Aβ.

1.6.2   Late-onset Alzheimer's disease (LOAD) and AD risk factors

Late-onset AD, sometimes referred to as sporadic AD represents by far the vast majority of AD cases (Maslow, 2010). Most of these individuals have no known AD-causing mutations *per se*, but do have abnormally high levels of cerebral Aβ, particularly oligomeric Aβ, which is thought to be highly predictive of eventual AD changes (Lue et al., 1999). Accordingly, the fact that most currently known EOFAD genes cause AD through abnormal production of Aβ has led to other Aβ-centered hypotheses, centered around the search for genetic causes of LOAD (reviewed by Bertram et al., 2010) --e.g. with potential effects on Aβ production, aggregation and clearance.

Two such "candidate genes" evaluated for genetic association with AD were APOE (encoding apolipoprotein E [apoE]) on chromosome 19q13 (Strittmatter et al., 1993) and SORL1 (encoding the sortilin-related receptor, low-density lipoprotein receptor class A repeat-containing protein) on chromosome 11q23-q24 (Rogaeva et al., 2007). In the case of the former, the ε4 allele of a 3 allele haplotype (composed of ε2, ε3, and ε4 alleles, which exhibit distinct biochemical properties at the protein level) leads to a dose-dependent increase in AD risk, by as much as ~4-fold compared to non-carriers. In contrast, the rarer ε2 allele seems to confer protective effects when inherited with the ε3 compared to homozygous ε3 carriers (Corder et al., 1994; Farrer et al., 1997; Gerdes et al., 2000). Functionally, apoE-dysfunction (reviewed by Kim et al., 2009; Vance and Hayashi, 2010) has been linked to several key aspects in the Aβ-centered AD hypothesis (Table 1-3; adapted from Bertram et al., 2010). As for SORL1, it was shown to direct trafficking of APP into recycling pathways, and that when underexpressed, APP is sorted into Aβ-generating compartments (reviewed in Lee et al., 2008b).

As valuable as the "candidate gene" approach has been, it suffers from a major drawback in that it is centered around a preconceived functional and/or positional hypothesis. Fortunately, the last five years have seen the advent of microarray technology (Sharon et al., 2010), which has revolutionized genetic research. It allows for the assessment of several hundreds of thousands of single-nucleotide polymorphism (SNPs) in a single experiment and to perform genome-wide association studies (GWAS) in a largely hypothesis-free manner. A number of GWAS have been performed to date in AD (reviewed by Bertram et al., 2010). In addition to confirming the ε4 allele of APOE as an AD risk-factor in carriers, they have uncovered several new potential AD susceptibility genes (Table 1-3). In 2009, two large GWAS from the UK (Harold et al., 2009) and France (Lambert et al., 2009) highlighted three novel AD genes: CLU (clustrin; also known as apolipoprotein J), CR1 (complement component 3b/4b receptor 1) and PICALM (phophatidylinositol binding clathrin assembly protein); all three currently rank at the very top of the Alzgene meta-analyses, directly behind APOE.

Functionally, these genes have all been implicated in a number of functions directly related to Aβ (Table 1.3). For example, in addition to being involved in Aβ clearance (DeMattos et al., 2004; Zlokovic, 1996) and aggregation (DeMattos et al., 2002; Thambisetty et al., 2010), clustrin has been reported to play a role in Aβ fibrillization (DeMattos et al., 2004; DeMattos et al., 2002), in regulating brain cholesterol and lipid metabolism (Gelissen et al., 1998) and in the inhibition of neuronal apoptosis (Nuutinen et al., 2009). CR1 is the main receptor for the complement C3b protein, a key inflammatory protein activated in AD (Khera and Das, 2009; Wyss-Coray et al., 2002). Several lines of evidence suggest that complement activation protects against Aβ-induced neurotoxicity and may promote the clearance of Aβ (Rogers et al., 2006; Wyss-Coray et al., 2002). Finally, PICALM is involved in clathrin-mediated endocytosis (Tebar et al., 1999), synaptic transmission and the removal of apoptotic cells (Harel et al.,

2008; Yao et al., 2005). With respect to AD, brain-expressed PICALM protein is predominantly found in endothelial cells, where it could facilitate Aβ clearance through the bloodstream (Baig et al., 2010). This hypothesis is supported by recent data that, similar to apoE ε4, the PICALM risk allele is associated with reduced levels of Aβ in the cerebral spinal fluid (CSF) of AD patients and normal individuals (Schjeide et al., in press).

In addition to the three genes above, we should point out that other GWAS have identified other potential AD genes, but independent replication of these findings have been inconsistent. These include GAB2 (GRB2-associated binding protein 2; Reiman et al., 2007), ATXN1 (ataxin 1), CD33 (siglec 3) and an uncharacterized locus of chromosome 14 (Ionita-Laza et al., 2007), and PCDH11X (protocadherin 11 X-linked; Carrasquillo et al., 2009).

Potential mechanisms linking genome-wide association findings in the pathogenesis of AD

	APOE	ATXN1	BIN1	CD33	CLU	CR1	GAB2	PCDH11X	PICALM
Aβ-production		Zhang et al., 2010	Wigge et al., 1997; Pant et al., 2009				Nizzari et al., 2007	Haas et al., 2005	Tebar et al., 1999
Aβ-aggregation	Kim et al., 2009a; Moir et al., 1999				DeMattos et al., 2002; Thambisetty et al., 2010				
Aβ-clearance	Kim et al., 2009a; Holtzman et al., 1999		Wigge et al., 1997; Pant et al., 2009		Zlokovic et al., 1996; DeMattos et al., 2004	Wyss-Coray et al., 2002; Rogers et al., 2006			Tebar et al., 1999; Baig et al., 2010
τ-phosphorylation							Reiman et al., 2007		
Synaptic transmission								Senzaki et al., 1999; Blanco et al., 2000	Yao et al., 2005; Harel et al., 2008
Inflammation	Kim et al., 2009a			Xie et al., 2005	Crocker et al., 2007; von Gunten and Simon, 2006	Wyss-Coray et al., 2002; Khera and Das, 2009			
Cerebrovascular events	Kim et al., 2009a								

Table 1-3. Overview of the potential functional impact of GWAS findings and their reported or suggested potential involvement in a number of pathogenetic pathways of relevance to AD

(Image adapted from Bertram et al., 2010)

1.6.3 Age

One notable aspect that routinely gets overlooked in AD is the age factor. As mentioned earlier, after the age of 65, the incidence and prevalence of AD doubles every 5 years. Moreover, even in carriers of risk-factor genes, the disease essentially does not occur in middle age. Therefore, whether or not an individual is genetically predisposed, aging remains an essential factor in AD (Hoyer, 1994), indicating perhaps that an age related component is involved in the development of the disease. Obviously, this age-related penetrance represents a significant risk factor in several other chronic diseases, including other neurodegenerative diseases, cancer, atherosclerosis among others, suggesting that there may exist common etiologies with diverse consequences.

1.7 Aβ aggregates and their role in AD

Aβ is widely considered to be at the heart of AD pathogenesis (reviewed by Nathalie and Jean-Noel, 2008). As discussed above, several Aβ species, ranging from 38 to 43 amino acids, are produced as a result of the sequential cleavage of APP by β- and γ-secretases. Of these Aβ species, some are considered more damaging than others, most notably Aβ42 due to its higher propensity to spontaneously self-aggregate into multiple coexisting physical forms (Burdick et al., 1992; Jarrett et al., 1993).

1.7.1 Oligomeric versus fibrillar Aβ

The ubiquitous nature of the fibrillar form of Aβ in senile plaques naturally led to the initial hypothesis that it caused neurodegeneration in AD (reviewed by Hardy and Selkoe, 2002). Indeed, over time, synthetic Aβ spontaneously aggregates into

neurotoxic β-rich fibrils, resembling those in plaques (reviewed by Gorman and Chakrabartty, 2001), further supporting the amyloid hypothesis. However, several studies have demonstrated that the number of SP in a particular region of the brain correlates poorly with the local extent of neuronal death, or synaptic loss, or with cognitive impairment (Dickson et al., 1995; Katzman, 1986; Terry et al., 1991). Instead, several recent studies have reported a robust correlation between the levels of soluble Aβ oligomers and the severity of the disease (reviews by Caughey and Lansbury, 2003; Ferreira et al., 2007; Haass and Selkoe, 2007; Klein et al., 2001).

1.7.2 Types of Aβ oligomers

The precise nature of the toxic Aβ oligomers remains contentious. They generally range from 2 to 6 peptides (Kayed et al., 2003; Lambert et al., 1998) but have been reported being as large as 12 peptides (Lesné et al., 2006) or even 24 in some cases (reviews by Glabe, 2008; Roychaudhuri et al., 2009). Interestingly, another form of Aβ has been isolated from the brains of AD patients, an N-terminally truncated Aβ (Aβ 3-40/42) bearing a pyroglutamate modification (Harigaya et al., 2000). Pyroglutamate-Aβ (pE-Aβ) as it is referred to, has been shown to have a higher propensity for oligomerization and aggregation than full-length Aβ, potentially seeding the accumulation of neurotoxic Aβ oligomers (reviewed by Gunn et al., 2010). Consistent with this idea, inhibition of glutaminyl cyclase was reported to reduce amyloidosis and ameliorate cognitive deficits in mouse models of AD (Schilling et al., 2008).

1.7.3   Mechanism(s) of toxicity of Aβ oligomers

Regardless of the exact conformation, soluble Aβ oligomers are, as a whole, decidedly more neurotoxic than fibrillar Aβ, and have been shown to inhibit a host of critical neuronal function, including long-term potentiation (LTP), synaptic dysfunction and memory loss in animal models and in cell culture (Lambert et al., 1998; Lesné et al., 2006; Shankar et al., 2008; Wang et al., 2002), through a mechanism(s) that remains unclear. Glabe *et al.* have postulated that multiple Aβ oligomer conformations may be produced through alternative pathways (Glabe, 2008) adding even more complexity to the puzzle (Figure 1-12). If that were the case, it is not inconceivable that each conformation acts *via* a specific set of neurotoxic pathways, which may account for the enormous diversity in the mechanisms that have been reported so far.

1.7.3.1   Extracellular mechanisms

The idea of a putative Aβ receptor is one that has garnered a number of advocates over the years. Although many have been put forward (Table 1-12A), a definitive candidate has so far failed to gain a consensus (reviews by Chow et al., 2009; Querfurth and LaFerla, 2010; Sakono and Zako, 2010). We will highlight some of the notable ones below.

Several studies have suggested that apoptotic cell death occurs through the interaction of Aβ oligomers with low-affinity NGF receptor [pan neurotrophin receptor (p75NTR)] and the activation of downstream effectors such as c-Jun N-terminal kinase (reviewed by Coulson, 2006). However, this remains controversial, as other studies have shown that p75NTR promotes neuronal survival and differentiation, suggesting perhaps a dual

role both in both cell death and cell survival (reviewed by Dechant and Barde, 2002). Additionally, other studies have reported conflicting evidence showing that p75NTR exerts in fact a protective role against Aβ oligomers (Costantini et al., 2005; Zhang et al., 2003).

Other studies have demonstrated that Aβ-derived diffusible ligands (ADDLs) bind to NMDA-type glutamate receptor (NMDAR) causing abnormal calcium homeostasis, leading to oxidative stress and ultimately to synapse loss (De Felice et al., 2007; Shankar et al., 2007). ADDLs can also induce the loss of insulin receptors in neurons (De Felice et al., 2009; Zhao et al., 2008) and impair LTP-associated kinase activity (Townsend et al., 2007).

The Frizzled (Fz) receptor has also been implicated in Aβ signaling (Magdesian et al., 2008). Inhibition of Wnt signaling by Aβ, relieves Fz-mediated inhibition of Gsk3β thereby increasing tau phosphorylation and neurofibrillary tangles, leading to cellular dysfunction. Yet another study found that Aβ oligomers can interact with synaptic proteins or channels and impair presynaptic P/Q-type calcium currents at both glutamatergic and gamma-amino butyric acid (GABA)-ergic synapses (Valincius et al., 2008). Recently, Lauren *et al.* reported that cellular prion protein (PrP^C) can act as an Aβ oligomer receptor with nanomolar affinity, mediating synaptic dysfunction (Lauren et al., 2009). Interestingly, although misfolded prion protein (PrP^{Sc}) is thought to cause prion disease, the interaction with Aβ does not require the infectious conformation. Finally, Aβ oligomers have been shown to destabilize the cell membrane (Valincius et al., 2008) and form pores which allow the abnormal flow of ions such as calcium into the neurons (Kawahara and Kuroda, 2000).

1.7.3.2   Intracellular mechanisms

Although Aβ was first identified as a component of plaques, as discussed above, ample evidence has demonstrated that it is generated intracellularly (reviwed by LaFerla et al., 2007). In addition to being produced intracellularly, previously secreted Aβ can be taken by cells and internalized through various receptors and transporters such as the nicotinic acetylcholine receptor, low-density lipoprotein receptor, formyl peptide receptor-like protein 1, NMDAR and the scavenger receptor for advanced glycation end-product (Figure 1-12B; reviews by LaFerla et al., 2007; Sakono and Zako, 2010). This has led many to propose an intracellular-mediated toxicity mechanism for Aβ oligomers. Unfortunately, the exact details of such a mechanism remain ambiguous.

Microinjections or over-expression of Aβ in primary neurons lead to the formation of low-molecular-weight Aβ oligomers accompanied by cell death (Chui et al., 2001) *via* the p53-BAX pathway (Zhang et al., 2002). A recent study by Mousnier *et al.* demonstrated a possible prefoldin (PFD)-mediated proteasomal protein-degradation pathway (Mousnier et al., 2007), leading to speculations that an Aβ-PFD complex could conceivably bind the proteasome complex and disrupt proteasomal function, causing eventual cell death; an idea that has received some traction in light of recent reports showing that proteasomal function was inhibited by interaction with Aβ oligomers, potentially leading to age-related accumulation of Aβ and tau protein (Tseng et al., 2008).

Figure 1-12. Formation and toxicity mechanisms of Aβ oligomers

(**A**) Formation and toxicity mechanisms of extracellular Aβ oligomers. Aβ is released extracellularly as a product of proteolytically cleaved, plasma membrane-localized amyloid precursor protein (APP). Extracellular Aβ oligomers can be formed in the presence of GM1 ganglioside on the cell membrane. GM1 induces Aβ oligomer-induced neuronal cell death mediated by nerve growth factor (NGF) receptors. Toxic non-fibrillar Aβ is also produced in the presence of αB-crystallin and ApoJ. A cellular prion protein (PrP^C) acts as an Aβ oligomer receptor with nanomolar affinity, and mediates synaptic dysfunction. Furthermore, the membrane pore is formed by Aβ oligomers. The pores allow abnormal flow of ions, such as Ca^{2+}, which causes cellular dysfunction. Binding of Aβ oligomers to the NMDA-type glutamate receptor (NMDAR) also causes abnormal calcium homeostasis, leading to increased oxidative stress and synapse loss. Binding of Aβ oligomers to the Frizzled (Fz) receptor can inhibit Wnt signaling, leading to cell dysfunctions such as tau phosphorylation and neurofibrillary tangles. Moreover, Aβ oligomer can induce insulin receptor loss from the neuronal surface and impaired kinase activity related to long-term potentiation. (**B**) Formation and toxicity mechanisms of intracellular Aβ oligomers. Aβ can be localized intracellularly by the uptake of extracellular Aβ or by the cleavage of APP in endosomes generated from the ER or the Golgi apparatus. Extracellular Aβ is internalized through various receptors and transporters, such as formyl peptide receptor-like protein 1 (FPRL1) or scavenger receptor for advanced glycation end-products (RAGE). These receptor–Aβ complexes are internalized into early endosomes. Most Aβ in the endosome is degraded by the endosome / lysosome system. However, Aβ in the lysosome can leak into the cytosol by destabilization of the lysosome membrane. Although cytosolic Aβ can be degraded by the proteasomal degradation system, inhibition of the proteasome function by Aβ oligomers causes cell death. Suppression of protein aggregation by interactions with various cellular proteins, such as prefoldin (PFD) or other molecular chaperones, may cause the formation of Ab oligomers. (Image adapted from Sakono and Zako, 2010)

1.8 Other APP metabolites and their role in AD pathology

1.8.1 sAPPα

As summarized earlier (Section 1.5.4), the beneficial properties of APP (pro-survival, trophic factor, memory formation, etc.) have been largely attributed to sAPPα, which is released after α-secretase cleavage of APP by one or more of the ADAM family of proteases, including ADAM9, ADAM10 and ADAM17. At the moment, it is unclear which of these is the relevant protease. However, over-expression of ADAM10 has been shown to reduce Aβ levels and plaque deposition in addition to ameliorating cognitive deficits in an AD mouse model (Postina et al., 2004), positioning ADAM10 as a good candidate for the relevant α-secretase in AD pathogenesis. Nevertheless, how sAPPα is related to AD remains a mystery. While sAPPα concentrations in CSF are reduced in carriers of the Swedish APP mutation (Lannfelt et al., 1995), those of sporadic AD cases have been reported unaltered (Olsson et al., 2003; Sennvik et al., 2000) or increased (Lewczuk et al., 2008). It is likely that the Dutch (APP E693Q) and Flemish (APP A692G) mutations similarly affect the α-secretase cleavage site of APP, reducing sAPPα (Kumar-Singh et al., 2000; Moechars et al., 1999). Interestingly, families with these mutations display a rather disparate clinical feature, with congophilic amyloid angiopathy and in the case of the Flemish mutation also AD-like dementia and neuropathology (Cras et al., 1998; De Jonghe et al., 1998). In these cases, both the altered Aβ sequence and reduced sAPPα may contribute to the clinical picture.

1.8.2   P3

To date, no clear biological or pathological role has been found for the P3 fragments ($P3_{40}$/$P3_{42}$) that are generated as a result of α- and γ-secretase cleavage of APP.

1.8.3   sAPPβ

Unlike sAPPα, sAPPβ has no demonstrable neuroprotective effect and its role in AD remains unclear. However, a recent report has shown that sAPPβ may serve as a precursor for an N-terminal fragment of APP (N-APP), which is thought to play a critical role in the pruning of axons (Nikolaev et al., 2009). In this study, N-APP was found to act as a ligand for the death receptor 6 (DR6; a member of the TNFα family), which upon binding, leads to activation of caspase-6. Caspase-6 activation was associated with degeneration of axons, but not of the cell body. The study suggests that APP and DR6 may be components of a neuronal self-destruction pathway, indicating that aberrant β-secretase cleavage of APP and production of N-APP may contribute to degeneration in AD. Other possible functions of sAPPβ may include suppression of neuronal stem cell differentiation in favor of glial differentiation (Kwak et al., 2006), but these have not been well characterized either from a physiological or pathological perspective.

1.8.4   AICD and C31

In addition to secretases, APP is subject to cleavage at its C-terminal end by caspases to release a second 31-amino-acid-long neurotoxic fragment, termed C31, as well as membrane-bound APPΔC31. Relatively little is known about the physiological role of

C31 in cells. Most of its speculated functions have been inferred from studies done on the APP intracellular domain. Two critical sites have been identified on the C-terminus of APP:

The first one is a threonine residue at position 668 (APP[695] numbering), which has been shown to be phosphorylated by several kinases, including cyclin-dependent kinase 5 (Cdk5), c-Jun N-terminal kinase 3 (Jnk3) and glycogen synthase kinase 3β (Gsk3β) (Iijima et al., 2000; Kimberly et al., 2005; Muresan and Muresan, 2005a). It is not clear what the role of this phosphorylation event is, but it has been implicated in the regulation of APP to the growth cones and neurites at the nerve terminals (Ando et al., 1999; Muresan and Muresan, 2005a, b). More importantly, it has been shown that the Threonine[668] phosphorylated fragments are increased in AD, but not in control brains, and that phosphorylation may facilitate BACE1 cleavage of APP (Lee et al., 2003). How this phosphorylation event relates to APP cleavage at D664 and C31 production remains unclear. Increased levels of C31 have been detected in AD compared to control brains (Zhao et al., 2003), but paradoxically, a recent report showed that APP phosphorylated at threonine[668] was less vulnerable to cytoplasmic caspase-cleavage at D664 (Taru et al., 2004).

The second critical site is a highly conserved domain found on all three APP family genes, which has been identified as the YENPTY motif. This motif has been shown to be required to allow interactions between the C-terminal of APP with various adaptor proteins, including Mint-1/X11a (and the family members Mint-2 and Mint-3), Fe65 (as well as Fe65-like proteins, Fe65L1 and Fe65L2) and c-Jun N-terminal kinase (JNK)-interacting protein (JIP), through the phosphotyrosine-binding (PTB) domain (Ando et al., 1999; Kimberly et al., 2005; Ramelot and Nicholson, 2001). Of particular interest is the interaction of APP C-terminal fragments with Fe65. APP intracellular C-terminal

domain (AICD) fragment (C57/C59) has been demonstrated to induce transcriptional activation in combination with Fe65 and Tip60, a histone acetyltransferase, using a reporter gene system (Cao and Sudhof, 2001). Interestingly, C31 was also shown to be able to induce expression of genes, and one of the genes identified was Gsk3β (Kim et al., 2003). In addition to driving Gsk3β expression, they demonstrated that both AICD and C31 could not only induce cell death, but could also induce phosphorylation of Tau (AT8) which are two hallmarks associated with AD. Several other studies have confirmed the toxic effects of C31 in wide array of cells, including PC12, N2a, neuronal and glial cells (Galvan et al., 2006; Kim et al., 2003; Lu et al., 2000). Interestingly, there is some debate as to whether or not it is C31 that specifically causes these effects or whether they are due to the cleaved C-terminus of APP (APPΔC31). There are published reports that cells devoid of APP, such as the B103 mouse neuroblastoma cell line, are inherently resistant to C31, but succumb to its toxic effects once wild type APP is transfected back in, with APPΔC31 having no effect (Lu et al., 2003b). However, another earlier study had reported that adenovirus mediated over-expression of APPΔC31 was sufficient to induce apoptosis in a variety of cell lines, including NT2, COS-1 and HeLa cells (Nishimura et al., 2002).

The most compelling argument for the link between AD and APP cleavage at aspartate[664] (D664) however, comes from studies performed on a modified transgenic APP mouse line (J20). These mice express a mutated form of APP, which, in addition to the Swedish and Indiana mutations, also carry a mutated C-terminus (D664A). Surprisingly, these mice exhibit none of the effects seen in their J20 counterparts and perform just as well as their wild-type littermates: LTP impairments are completely blocked, cognitive and behavioral deficiencies are completely absent, and astrogliosis and more importantly dendritic spine loss are significantly abrogated. Interestingly, all of these effects are prevented in spite of unaltered levels of secreted Aβ, Aβ deposition

and plaque load (Galvan et al., 2006; Saganich et al., 2006). These results suggest that D664 cleavage of APP may be, at least in part, responsible for disrupting cellular signals controlling synaptic integrity. Concordantly, a recent report from the Bredesen laboratory has revealed a significant increase in nuclear p21-activated kinase (isoforms 1,2 and 3; PAK1/2/3) activation in the hippocampus of 3 month old J20 mice, compared to non-transgenic littermates, an effect that is completely prevented in the modified J20 D664A mice. In contrast, 13 month old J20 mice displayed a significant decrease in PAK1/2/3 activity, which was not observed in the J20 D664A mice (Nguyen et al., 2007). These findings are consistent with the hypothesis that dysregulation of certain signaling pathways observed in AD, may in fact require caspase-mediated cleavage of APP at D664, specifically in the case of PAK signaling, which interestingly is an important modulator of dendritic spine maintenance, downstream of the Rho GTPase, Rac1.

CHAPTER 2: PERSPECTIVES AND OBJECTIVES OF THE PRESENT STUDY

2.1 Is APP a receptor?

When the amyloid precursor protein (APP) was cloned in 1987 (Kang et al., 1987), it was originally observed that APP possessed many of the characteristics of a typical transmembrane receptor (discussed earlier), which raised the possibility that APP could initiate a signal transduction cascade through an unidentified ligand. To date, the evidence to support this idea remains scarce, as no clear and definitive candidate has emerged as the major ligand responsible for triggering an APP-mediated signaling cascade. Nevertheless, several molecules have been found to bind the ectodomain of APP; these include metal ions such as copper and zinc (Bush et al., 1993; Hesse et al., 1994), as well as several components of the extracellular matrix, such as collagen (types I and IV), heparan sulfate proteoglycan, heparin, laminin and glypican (Beher et al., 1996; Caceres and Brandan, 1997; Williamson et al., 1996), among several others. As we summarized earlier, the discovery of these binding partners lend support to many of the proposed physiological functions for APP --e.g. to facilitate the transport of metal ions or to promote cell to cell adhesion (reviewed by Thinakaran and Koo, 2008). Interestingly, it was discovered that the cell-adhesion-promoting functions of APP were mediated through intercellular (trans-configuration, Figure 2-1B) homo- and hetero- complexes of the APP family proteins (Soba et al., 2005). Furthermore, in addition to the intercellular interactions, it was found that the APP and APLP2 proteins could also interact in an intracellular manner (cis-configuration, Figure 2-1A).
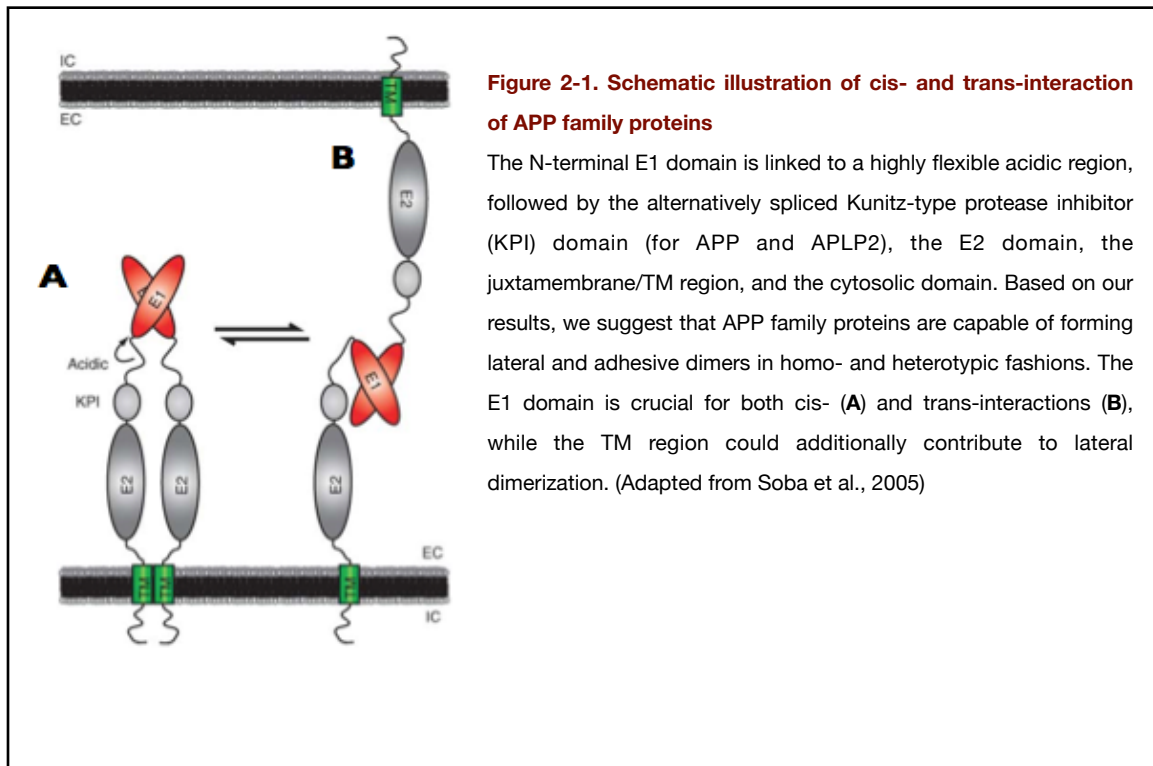
The physiological significance of this latter type of interaction has so far remained elusive. However, it has been well described that in mammalian cells, the most common mechanism of signal transduction activation for single-pass transmembrane proteins occurs through a ligand-mediated shift in the monomer/dimer equilibrium (reviewed by Ullrich and Schlessinger, 1990). Indeed, in addition to monomeric proteins, dimeric and tetrameric forms have been isolated for both isolated and full length APP (Gralle et al., 2006; Scheuermann et al., 2001; Wang and Ha, 2004). This has led many to hypothesize that the receptor function of APP and its ability to trigger a signal transduction cascade may be linked to its oligomerization state. More importantly, several pieces of data have suggested that disruption of the oligomerization state of APP could play a role in the pathology of AD (Gralle et al., 2009; reviewed by Khalifa et al., 2010; Soba et al., 2005). Indeed, evidence of dimerization of proteins as a pathological mechanism in neurodegenerative processes has been demonstrated in current models of the prion protein's (PrP) role in scrapie pathogenesis, where

oligomeric forms of PrP are believed to facilitate a more rapid conversion of PrP^c to PrP^{sc} (Priola et al., 1995; Turk et al., 1988). Could a similar mechanism be in play in AD pathogenesis as well? Is APP at the center of a pathogenic loop?

2.2   Why does Aβ increase with aging and is APP signaling a factor?

It is generally believed that both EOFAD and LOAD share a common pathological mechanism, with the Aβ peptide being at the center of it all. With the discovery of the many autosomal dominant mutations in APP and presenilins, we have a fairly detailed understanding of why Aβ levels are elevated in EOFAD. However, in LOAD, in spite of considerable advances in understanding the pathogenesis of AD, one critical question continues to pose a significant challenge: why do Aβ levels, especially oligomeric Aβ, increase in the brains of AD patients that have no mutations in the APP or secretase genes? As we have discussed above (Section 1.7.2), the discovery of several risk-factor genes has shed some light on the matter, illustrating several potential pathways leading to an imbalance in the levels of Aβ. However, as we have also pointed out, since age remains by far the highest risk factor for AD, what is the link between these two factors, i.e. aging and Aβ levels?

It is often argued, supported by a wealth of evidence (reviewed in Zhang and Xu, 2007), that the elevated levels of Aβ in AD are due to increased processing by either or both of the secretases, notably β- and γ-secretases. Yet, maintaining physiological levels of Aβ requires a finely tuned equilibrium between two seemingly independent processes: production and clearance (reviewed in De Felice and Ferreira, 2002). This is the basis for many alternative suggestions that the abnormal increases in cerebral Aβ may be completely independent of the direct metabolism of APP itself. For example many have

hypothesized that increases in Aβ levels represent rather a deficiency in the proteases that physiologically degrade Aβ, such as neprilysin and insulin-degrading enzyme (IDE), allowing the accumulation of the peptide (Farris et al., 2007; Selkoe, 2001).

Alternatively, the tight relationship between trafficking and processing of APP have raised the possibility of a link between cholesterol levels and LOAD, supported by the discovery of the lipid transport protein apoE as a risk factor for AD (allele ε4). As an illustration of that idea, lowering plasma cholesterol levels with statins was reported to contribute to decreasing the risk of dementia (Jick et al., 2000). Cholesterol could affect the trafficking and the affinity of APP for the secretases, thereby influencing the release of Aβ (Kojro et al., 2001). Cerebral trauma is also a known risk factor for dementia (Jellinger, 2004), raising the possibility that short-term post-injury regenerative processes could lead to long-term neurodegeneration. In this regard, a relevant observation is that traumatic brain injuries in mice directly lead to increased --perhaps adaptive-- expression of APP and to the deposition of Aβ (Blasko et al., 2004; Roberts et al., 1991; Roberts et al., 1994). Finally, in different mouse models, deficiencies in trophic factors, such as nerve growth factor (NGF) have been reported to cause neuronal degeneration and increase production of Aβ as a secondary consequence (reviewed in Isacson et al., 2002).

Ultimately, whether it is driven by cholesterol imbalance or by traumatic brain injuries, it is clear that several factors directly or indirectly affect APP processing and Aβ production. Moreover, the evidence strongly suggests that the increased Aβ burden plays a causative role in dementia, specifically AD. This raises an important question: does APP signaling affect APP processing and Aβ production?

2.3   Aims of the current study

In the chapters that will follow, we will describe a series of experiments which were designed to investigate whether APP signaling plays a pathogenic role in Alzheimer's disease. We will present evidence suggesting that aberrant signaling through dimerization of cell-surface APP may play an important role in the pathogenesis of Alzheimer's disease (AD). Our data will show that dimerization of APP is sufficient to affect survivability and to alter normal synaptic functions in hippocampal neurons. Additionally, we will demonstrate that dimerization of APP may be an important factor in how the processing of APP itself is regulated. We will show that dimerization of APP in hippocampal neurons is sufficient to initiate amyloidogenic processing of APP, resulting in increased production of the β-amyloid peptide.

CHAPTER 3: MATERIALS AND METHODS

3.1   SDS-PAGE and Western blotting

After treatments, cells were rinsed twice with ice-cold PBS and harvested in 1x RIPA buffer (Pierce; Rockford, IL), supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktails (Pierce; Rockford, IL). Samples were incubated on a shaker for 20 minutes at 4°C and then briefly sonicated on ice before being centrifuged for 20 minutes at 14K rpm at 4°C. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce; Rockford, IL) according to the manufacturer's protocol.

For SDS-PAGE, cell lysates containing 20-50 µg of total protein were boiled for 5 minutes and loaded on pre-cast NuPAGE® Novex 4-12% Bis-Tris gels (Invitrogen; Carlsbad, CA). Proteins were transferred onto PVDF or nitrocellulose membranes (Millipore Corporation; Bedford, MA). Primary antibodies were diluted in SuperBlock® blocking buffer in TBS (Pierce; Rockford, IL) containing 0.1% (v/v) Tween® 20 (Sigma; St Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies [goat anti-mouse-HRP, goat anti-rabbit-HRP (1:3000; Cell Signaling Technology; Beverly, MA) and donkey anti-goat-HRP (1:2000; Santa Cruz Biotechnology, Inc; Santa Cruz, CA)] were diluted in TBS-T [50 mM Tris, pH 8.0; 150 mM NaCl and 0.1% (v/v) Tween® 20] containing 5% (w/v) non-fat dry milk (Bio-Rad Laboratories; Hercules, CA). Chemiluminescence was detected using Immobilon™ Western chemiluminescent HRP substrate (Millipore Corporation; Bedford, MA). Autoradiography was carried out using Biomax™ MR films (Eastman Kodak; Rochester, NY).

3.2   Densitometry and statistics

All Western blot scans and DNA gel images were quantified using the free image processing and analysis software Image J (National Institute of Health; Bethesda, MD). Statistical analysis was performed using the statistics package GraphPad Prism®.

3.3   Antibodies

Anti-APP N-terminal mouse antibody (mAb) 22C11 (1:4000; Millipore Corporation; Bedford, MA); anti-Aβ mAb 4G8 (1:4000; Millipore Corporation; Bedford, MA); anti-APP C-terminal mAb (1:1000; Millipore Corporation; Bedford, MA); anti-Aβ mAb 6E10 (1:2000; Millipore Corporation; Bedford, MA); anti-APPΔC31 rabbit antibody (rAb) [1:2500; a gift from E. Koo at the Scripps Institute in La Jolla, CA (Galvan et al., 2002)]; anti-PSD-95 rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-Drebrin A rAb (1:1000; Sigma; St Louis, MO); anti-BACE1 rAb (1:1000; Abcam; Cambridge, MA); anti-GGA3 rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-caspase-3 rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-cleaved-caspase-3 rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-sAPPβ mAb (1:4000; Millipore Corporation; Bedford, MA); anti-IDE rAb (1:1000; Abcam; Cambridge, MA); anti-neprilysin rAb (1:1000; Millipore Corporation; Bedford, MA); anti-ERK1 rAb (1:1000; Santa Cruz Biotechnology, Inc; Santa Cruz, CA); anti-GAPDH mAb (1:1000; Abcam; Cambridge, MA); anti-dendra2 rAb (1:1000; Evrogen; Moscow, Russia); anti-cleaved-Notch rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-Aph-1 rAb (1:1000; Santa Cruz Biotechnology, Inc; Santa Cruz, CA); anti-HA-tag rAb (1:1000; Cell Signaling Technology; Beverly, MA); anti-EEA1 mAb (1:1000; Abcam; Cambridge, MA); anti-PS1 mAb (1:1000; Abgent; San Diego, CA); anti-βIII-tubulin mAb TUJ1 (1:1000; Covance; Princeton, NJ).

3.4   Hippocampal neuron cultures

Hippocampal neuron cultures were prepared following a slightly modified version of the previously described Brewer method (Brewer et al., 1993). Briefly, fetuses at embryonic day 18 (E18) from timed pregnant Sprague-Dawley rats (Taconic Farms; Hudson, NY) were sacrificed and the hippocampi removed and collected in room temperature Hank's balance salt solution (HBSS-; Invitrogen; Carlsbad, CA), supplemented with 0.6% (w/v) glucose (HBSS+). The hippocampi were then incubated in 0.25% trypsin (Invitrogen; Invitrogen; Carlsbad, CA) for 15 minutes at 37°C and washed (10 minutes, x3) with HBSS+. Finally, the neurons were dissociated in Neurobasal® medium (Invitrogen; Carlsbad, CA) supplemented with B27 supplements and Glutamax™-1 (Invitrogen; Carlsbad, CA), and plated at a density of $2.5x10^5$ cell/ml on dishes coated with poly-L-lysine (Sigma; St. Louis, MO). The resulting neuronal cultures consists of a population enriched in large pyramidal neurons that constitute the main initial target in AD pathogenesis. For our experiments, neurons were used after approximately 14-21 days *in vitro* (DIV).

3.5   Cell-line cultures

Human embryonic kidney 293 (HEK 293) and murine neuroblastoma B103 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM; I Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Carlsbad, CA) and 10% FBS + 5% horse serum (HS; Invitrogen; Carlsbad, CA) respectively. For stably transfected B103 cells expressing APP, the media was supplemented with 50 µg/ml Geneticin (Invitrogen, Invitrogen; Carlsbad, CA).

3.6   Preparation of Aβ oligomers

Aβ oligomers were prepared as previously described by Barghorn (Barghorn et al., 2005). Briefly, lyophilized synthetic Aβ$_{42}$ peptide (American Peptide; Sunnyvale, CA) was allowed to equilibrate to room temperature for 30 minutes and resuspended in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma; St. Louis, MO) to 1.0 mM using a glass gas-tight Hamilton syringe with a Teflon plunger. After evaporating the HFIP in the hood, the resulting clear peptide film was dried under vacuum in a SpeedVac® for about 1 hour. The peptide was then resuspended to 5.0 mM in anhydrous dimethylsulfoxide (DMSO; Sigma; St. Louis, MO) by pipette mixing followed by bath sonication for 10 minutes and aliquoted. Each 5.0 mM Aβ$_{42}$ aliquot in DMSO was diluted with cold Neurobasal medium to a final concentration of 100 µM and incubated at 4°C for 24 hours. The preparation was centrifuged at 3000g for 20 minutes at 4°C before using.

3.7   Peptide and oligonucleotide delivery into cells

Peptides were synthesized and purified at the Stanford University Proteins and Nucleic Acid (PAN) Facility. Oligonucleotide sequences were custom ordered (Dharmacon; Lafayette, CO) with a thiol functionality at the 5'-end. Peptide and ologonucleotide stocks were solubilized in water at 1.0 or 10 mM concentration. The delivery peptide derived from the Drosophila Antennapedia homeodomain (CRQIKIWFQNRRMKWKK), also called penetratin-1™ (MP Biomedicals; Irvine, CA), was cross-linked *via* a Cys-Cys bond to the desired peptide or the oligonucleotide as previously described (Davidson et al., 2004).

3.8   Peptide and oligonucleotide sequences

C31 sequences: AAVTPEERHLSKMQQNGYENPTYKFFEQMQNC [C31]; AAVTPEERHLSKMQQNGAENATYKFFEQMQNC [G681/P685; C31m]; APP siRNA: 5'-UUGGCCAAGACAUCGUCGGdAdG-3' [si1]; 5'-

UGCAUUUGCUCAAAGAACUTdG-3' [si2]; 5'-UGCAGUUGCGCAACGAACUTdG-3' [APP mis-match]. Caspase-3 siRNA:

3.9 Generation of Fab fragments of 22C11

Fab fragments of 22C11 were prepared using the Pierce Fab micro preparation kit (Pierce; Rockford, IL) following the manufacturers instructions.

3.10 Quantification of Aβ by ELISA

Media and lysates were collected from treated and control hippocampal neurons and centrifuged for clarification at 14K rpm for 2 minutes at 4°C prior to analysis. Levels of Aβ were determined using an ELISA kit (Covance; Princeton, NJ) according to the manufacturer's protocol.

3.11 DiOlistic labeling of neurons

3.11.1 Preparation of gene gun bullets

This protocol was adapted from Gan and Grutzendler (Gan et al., 2000). Briefly, 100 mg of tungsten particles (1.1 μm diameter; Bio-Rad Laboratories; Hercules, CA) were thoroughly precipitated with 5.0 mg of lipophilic dye (DiI, DiO or DiD; Invitrogen; Carlsbad, CA), and dissolved in 100 μl of methylene chloride. For single labeling of neurons, only DiO was used. After drying on the glass slide, the particles were collected in a test tube with 3 ml dH$_2$O and subsequently placed in a sonicating water bath for 20 minutes. The solution was vortexed for a few seconds and injected into Tefzel tubing (Bio-Rad Laboratories; Hercules, CA) previously treated with 10 ml polyvinylpyrrolidone (PVP360; Sigma; St. Louis, MO) to improve particle

attachment to the tubing. The tube was then inserted into a tubing preparation station (Bio-Rad Laboratories; Hercules, CA) and after a 30-minute rotation period, the solution was slowly withdrawn. The particle-coated tube was then rotated and air-dried under a constant nitrogen flow (0.4 liter/min) for a further 30 minutes or until the tubing was dry. The tube was cut into small bullets (13 mm) and stored in a desiccated container at room temperature for up to one month.

3.11.2 Delivery of dye-coated particles

Dye-coated particles were shot into the cells using the Helios gene gun system (165-2431; BioRad Laboratories; Hercules, CA). To aid dye dispersion and prevent large particles from landing on the cells, a custom fabricated filter holder using a membrane with 3 µm diameter holes (TSTP04700; Millipore; Corporation; Bedford, MA) was placed between the gun and the cells. The cells were shot 2 to 3 times, depending on their density at a constant pressure of 150 psi, and left in 0.1 M PBS overnight to allow dye diffusion along neuronal processes. Cells were post fixed with 4% paraformaldehyde (PFA; Pierce; Rockford, IL) for 1 hour to preserve staining then mounted onto glass slides using Gel Mount^{TM} (Biomeda Corporation; Forster City, CA) and stored at 4°C in the dark.

3.11.3 Spine imaging

Labeled neurons were imaged using the Laser Scanning Microscope (LSM) 510 Meta confocal microscope (Zeiss), equipped with 40X 1.3 NA and a 100X 1.4 NA oil immersion objectives. Scanning used three excitation lines of the argon laser (488 nm for DiO, 568 nm for DiI and 647 nm for DiD) with three separate barrier filter sets (522 ± 35 nm for DiO, 580 ± 32 nm for DiI, and 680 ± 32 nm for DiD). Z stacks were collected at 1 µm intervals to cover the full depth of the neuronal dendritic trees (20, 30 µm) and then compressed into a single image.

3.11.4 Spine analysis, quantification and statistics

The NIH image software program Image J was used the quantify DiO labeled cultured neurons. An average of 8 compressed images (20 µm thick) consisting of pyramidal neurons in the hippocampus were quantified for each treatment. The NIH image software program gave two parameters: Number of puncta per 100 µm of dendrite and total dendrite area. Puncta included both spines and boutons along and around the dendritic trees of neurons. Cell bodies were blocked out so that only spines and dendrites were quantified. Statistical analysis was performed using the statistics package GraphPad Prism®.

3.12   Assessment of cell death

Cell death was assayed using the LIVE/DEAD® Cell Viability Assay kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. After the appropriate treatments, cells were treated with a solution containing 2.0 µM calcein AM and 4.0 µM Ethidium homodimer 1 (EthD-1) in PBS for 30 minutes. After washing off the solution, cells were directly analyzed without fixing. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells.

3.13   γ-Secretase activity assay

γ-Secretase activity was assayed adapting the previously described protocols (Hansson et al., 2006). Briefly, γ-secretase activity in hippocampal neurons was detected as AICD-myc formation using as substrate C99-myc derived from HEK293 cells transfected with a C99-myc plasmid (Appendix A). CHAPS-solubilized (20 mM HEPES, pH 7.0; 150 mM KCl; 2.0 mM EGTA; 1.0% CHAPS and 2x protease inhibitor cocktail) HEK293 cells were incubated together with solubilized 22C11-treated and untreated hippocampal neuron membranes (1:1 ratio) at -20°C or 37°C with or without the γ-secretase inhibitor (DAPT; 1.0 µM) for 2 hours. AICD fragments were detected by Western blot analysis using an anti-myc rAb (Cell Signaling Technology; Beverly, MA).

3.14   NucView caspase-3 activity assay

Caspase-3 activity in hippocampal neurons was assessed with the NucView^{TM} 488 Caspase Detection kit (Biotium Inc.; Hayward, CA) following the manufacturer's protocol. Briefly, hippocampal neurons cultured in chamber slides were treated with a 5.0 µM solution of the NucView caspase-3 substrate for 30 minutes. Cells were then fixed with 4.0% paraformaldehyde (Pierce, Rockford, IL), mounted and observed under a fluorescence microscope using a fluorescein isothiocyanate (FITC) filter.

3.15   Immunocytochemistry

Hippocampal neurons grown in chamber slides were treated with 22C11 (100 ng/ml) only or 22C11 (100 ng/ml) and Penetratin-1-linked-siCasp3 (80 nM) for 8 hours. After fixing with 4.0% paraformaldehyde (Pierce, Rockford, IL) cells were permeabilized with 0.5% Triton® X-100 (Sigma; St. Louis, MO) in PBS and incubated the anti-BACE1 rAb (1:1000; Abcam; Cambridge,

MA) and anti-EEA1 mAb (1:1000; Abcam; Cambridge, MA) overnight. Anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa® Fluor dyes (1:1000; Invitrogen; Carlsbad, CA) were used. Labeled neurons were imaged using the Laser Scanning Microscope (LSM) 510 Meta confocal microscope (Zeiss), equipped with 40X 1.3 NA and a 100X 1.4 NA oil immersion objectives. Scanning used two excitation lines of the argon laser (488 nm for Alexa Fluor-488, 568 nm for Alexa Fluor-568). Z stacks were collected at 1.0 μm intervals and then compressed into a single image for analysis using the Volocity® Imaging software (Volocity® Acquisition and Volocity® Visualization; Perkin-Elmer; Waltham, MA).

3.16   Isolation of cell-surface proteins

Cell surface proteins were biotinylated using the EZ-Link® NHS-PEO4 reagent (500 μg; Pierce; Rockford, IL) according to the manufacturer's protocol. Biotinylated proteins were precipitated using Dynabeads® M-280 Streptavidin beads (Invitrogen; Carlsbad, CA). Biotinylated proteins were resolved by SDS-PAGE and immuno-blotted with the appropriate antibodies.

3.17   Cross-linking of cell-surface APP with 22C11

HEK293 cells (6-well dish; ~10^6 cells) were transiently transfected with 4.0 μg of APP-dendra2 plasmid (Appendix A) with Lipofactamine^{TM} 2000 reagent (Invitrogen; Carlsbad, CA). 48 hours after transfections, the culture medium was removed and replaced with a 5.0 μg/ml solution of 22C11 and incubated at 4°C. After 2 hours, the solution was removed, the cells washed with ice-cold PBS and incubated with the cross-linker DTSSP (25 mM; Pierce; Rockford, IL) for 1 hour at 4°C. After quenching the reaction with 1 M Tris-HCl, cells were washed with ice-cold PBS (x3) and harvested in RIPA buffer (2-wells were pooled for each set of conditions). Cross-linked APP was immunoprecipitated and immuno-blotted with anti-dendra2 rAb.

3.18   Cycloheximide degradation time-course assay

Hippocampal neurons were treated with cycloheximide (CHX; 150 μM) only or with 22C11 (100 ng/ml) and CHX (150 μM) over an 18-hour time course. Lysates from each time time were immuno-blotted with the specific antibody for BACE1 (1:1000; Abcam; Cambridge, MA).

3.19   qRT-PCR

To quantify mRNA levels, mRNA from 22C11-treated and untreated cells was isolated using the TRIzol® plus reagent (Invitrogen; Carlsbad, CA). Reverse transcription was carried out using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, CA). Real-time PCR was done using the SYBR Green method. The following primer pair for BACE1 were used F: 5'-CAGCTCTGTGGCGCTGGCTT-3' R: 5'-CCAGCACACCAGCTGCTCCC-3'.

3.20   Transfections and plasmids

Plasmids (sequences in Appendix A) used in these studies include the following: pcDNA3.1-dendra2 was a kind gift from Jordi Magrane at Cornell Universiry; pSG5L-Flag-HA-GGA3 (11185; Addgene; Cambridge, MA) was originally cloned by the Sellers Laboratory (Harvard university; Boston, MA). Wild-type C99 cDNA was cloned into pcDNA3.1 (Invitrogen; Carlsbad, CA) with a Myc-tag fused at the C-terminal end of C99 (Appendix A).

CHAPTER 4: NEUROTOXIC AND SYNAPTOTOXIC EFFECTS OF THE DIMERIZATION OF CELL-SURFACE APP IN HIPPOCAMPAL NEURONS

4.1   Introduction

In spite of the wealth of evidence pointing towards the existence of naturally occurring multimeric forms of APP on the cell surface (Gralle et al., 2006; Scheuermann et al., 2001; Wang and Ha, 2004), their physiological function(s) remain unclear. So far, the aggregation of cell-surface APP and APLPs has only been implicated in cell-cell binding (Soba et al., 2005) but, being a type I trans-membrane protein, APP has also been postulated to be involved in signal transduction (reviewed by Ullrich and Schlessinger, 1990). Unfortunately, while many molecules and proteins have been shown to bind to APP (Section 2-1), it is not known whether or not they influence the monomer/multimer equilibrium of cell-surface APP nor the pathways that are activated either in a physiological or a pathological context, such as in AD. Interestingly, soluble and insoluble aggregated forms of the Aβ peptide itself have been proposed as a potential ligand for APP in a pathologic context, able to induce apoptosis in cells upon their interaction at the cell surface (Lorenzo et al., 2000; Lu et al., 2003a; Shaked et al., 2006). While the exact mechanism is not known, it has been proposed that APP-dependent Aβ toxicity involves the facilitation of APP multimerization at the cell surface by aggregated Aβ. Indeed, cells lacking APP are resistant to the neurotoxic effects of Aβ (Lu et al., 2003b; White et al., 1998). In this proposed mechanism, aggregated Aβ interacts directly with cell-surface APP to facilitate APP homo-oligomerization by binding to its homologous sequence (amino acids 597-624 of APP[695]; Figure 1-8C) on the extracellular domain of APP (Lu et al., 2003a; Shaked et al., 2006). However, the specificity of Aβ binding to APP remains a controversial subject. As we reviewed earlier,

a wide variety of cell-surface proteins have been identified as not only binding partners for Aβ, but as being required for its neurotoxic effects (reviewed in section 1.7.3.1).The promiscuous nature of Aβ binding has made isolating the specific signaling pathway(s) triggered by Aβ binding to APP a challenging endeavor.

In order to bypass the absence of a known ligand for APP and to overcome the high propensity of Aβ to bind other receptors, we opted for an antibody-mediated approach to study APP signaling in neurons. The bivalent nature of most antibodies (Figure 4-1A) makes them ideal candidates to facilitate multimerization of receptors at the cell-surface, such as APP (Figure 4-1B). This approach afforded us two key advantages. First, no chemical modifications of APP were required, allowing us to study the effects of endogenous APP in its intact native form. Second, using an APP antibody allowed us to target a specific region of the APP molecule, notably the E1 domain, which has been shown to be important for its dimerization (Soba et al., 2005). In the present chapter, we show the effectiveness of the commercially available mouse monoclonal antibody directed against amino acids 66-81 of the E1 domain of the N-terminus of APP (clone 22C11; Hilbich et al., 1993) to alter the state of surface APP multimers in cultured cells. We demonstrate that this antibody triggers apoptotic death at high concentrations and synaptic alterations at low concentrations in hippocampal neurons, in an APP dependent manner. Our data suggests that dimerization of cell-surface is sufficient to trigger one or more signaling pathways ending in cell death and synaptic dysfunction in hippocampal neurons, indicating that APP signaling may play an important role in AD pathogenesis.

Figure 4-1. Strategy for inducing dimerization of APP in cultured cells

(**A**) Schematic representation of the general structure of divalent antibodies, with two antigen-binding sites. (**B**) Illustration showing the dimerization of APP at the cell surface by the divalent antibody directed at amino acids 66-81 in the E1 domain of the N-terminal end of APP.
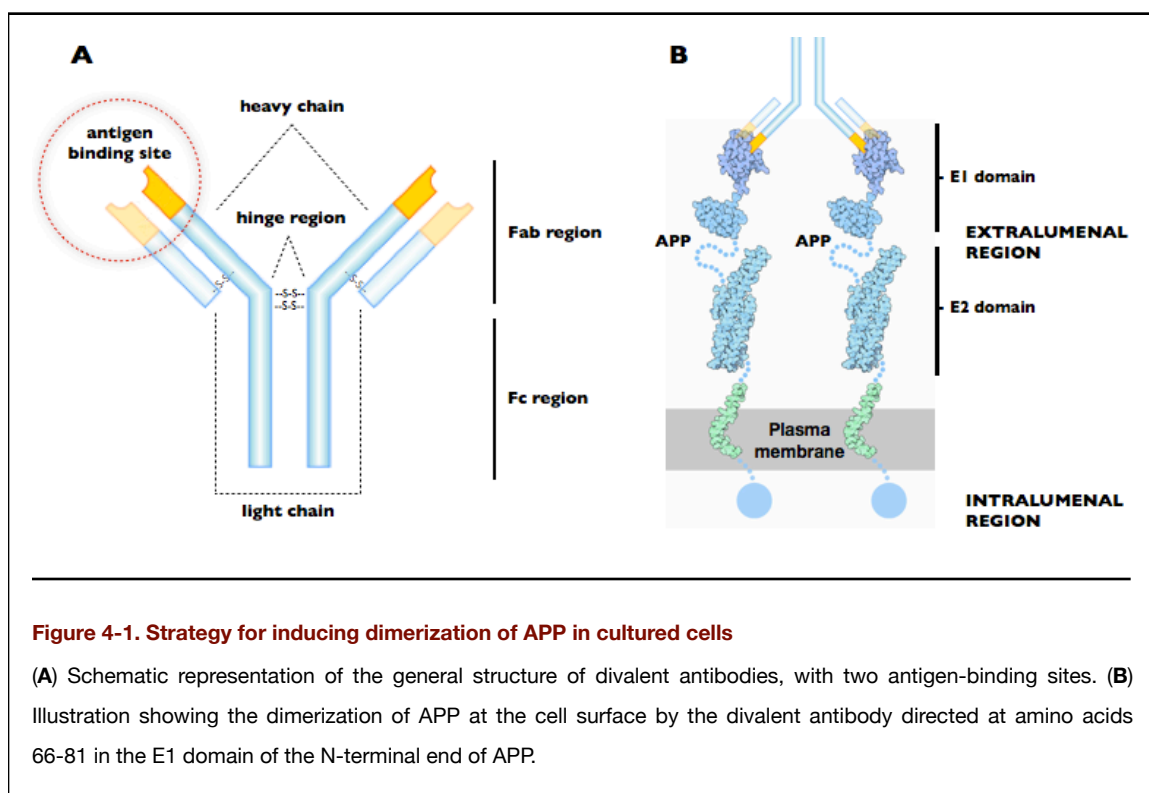
4.2 Results

4.2.1 Divalent APP antibody 22C11 increases the sub-population of dimeric and tetrameric APP expressed on the cell surface

Our first objective was to examine whether the APP N-terminal antibody 22C11 could alter multimerization of APP at the cell surface. Low levels of surface APP in neurons forced us to employ an APP over-expressing model for those studies. B103 cells were transiently transfected with an APP construct, fused at its C-terminus with the fluorescent protein dendra2 (Gurskaya et al., 2006) to facilitate immunoprecipitation of the APP complexes with an anti-dendra2 antibody. Two days after transfection (Figure 4-2C), the cells were incubated with 22C11 (5.0 µg) for two hours, followed by incubation with the homobifunctional, thiol-cleavable and membrane impermeable

cross-linker 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) in order to cross-link and stabilize any APP multimers within 8 Å of each other. Isolation of cross-linked APP by immoprecipitation followed by Western blotting analysis (Figure 4-2A) with the same dendra2 antibody, revealed an increase not only in dimeric but in tetrameric complexes of APP in cells incubated with 22C11 compared to control cells incubated with PBS (Figure 4-2B). Consistent with previous reports (Gralle et al., 2009; Scheuermann et al., 2001), control cells also contained isolatable APP complexes in dimeric and tetrameric forms, suggesting that at least a sub-population of APP may exists as dimer and tetramers. To ascertain the identity of these higher molecular weight species, the immunoprecipitated complexes were treated with the reducing agent dithiothreitol (DTT) to cleave the cross-linker and release the cross-linked molecules. DTT treatment resulted in the disappearance of both upper bands, ~250 kDa and ~400 kDa (Figure 4-2A), corresponding to dimeric and tetrameric APP respectively.

These results suggest that the N-terminal antibody 22C11 is capable of increasing oligomerization of cell surface APP *in vivo* in cultured cells. We could not test the antibody in cultured hippocampal neurons, but given the previously published reports of antibody-mediated dimerization of cell-surface receptors (Gralle et al., 2009; Mbebi et al., 2002), we felt confident that 22C11 was a viable means of increasing oligomerization of cell-surface APP in cultured neurons as well.

Figure 4-2. Increased oligomerization of surface APP by the N-terminal antibody 22C11

B103 cells over-expressing APP-dendra2 were treated with N-terminal APP antibody 22C11 (5.0 µg) for 2 hours, followed by cross-linking with the dithiol cleavable cross-linker DTSSP. Immunoprecipitation of cross-linked APP complexes showed in enrichment in not only APP dimers, but of APP tetramers as well compared to control cells that were treated with PBS. (**A**) Representative Western blot showing increased APP dimers (~250 kDa) and tetramers (~400 kDa) after 22C11 incubation. The corresponding bands disappear after a 10 minute treatment with DTT prior to loading the gel (*non-specific band). (**B**) Densitometric analysis of the data was quantified from at least 3 independent experiments and expressed as the mean+/-SEM (p=0.06 for APP dimers, **p=0.035 for APP tetramers) band intensity. (**C**) Representative Western blot showing the expression levels of APP-dendra2 in transfected B103 cells.

4.2.2   Aβ42 induces dose-dependent neuronal death and synaptic dysfunction in hippocampal neurons

Recent evidence has suggested that Aβ oligomers are perhaps the main culprits in the pathology of AD (reviews by Caughey and Lansbury, 2003; Ferreira et al., 2007; Haass and Selkoe, 2007; Klein et al., 2001). Oligomeric preparations of synthetic $Aβ_{42}$ (Figure 4-3A) are highly toxic to hippocampal neurons at high concentrations (10 µM), reducing

the number of viable cells by ~50% after 24 hours of treatment (Figure 4-3B) and triggering marked neuritic dystrophy (Figure 4-3C). At low sub-apoptotic concentrations (300 nM), oligomeric $A\beta_{42}$ triggers synaptic alterations after 24 hours in hippocampal neurons, characterized by a significant decrease by as much as ~55% in the density of dendritic spine (Figure 4-4A, B), as well as a reduction in the protein levels of the post-synaptic markers PSD-95 (Figure 4-4C, D) and Drebrin A (Figure 4-4E, F), by ~30% and ~40% respectively, both of which have been shown to be critical for proper dendritic spine maintenance and synaptic function (Ehrlich et al., 2007; Ivanov et al., 2009; Mizui et al., 2005; Takahashi et al., 2006).

Figure 4-3. Neurotoxic effects of Aβ oligomers on hippocampal neurons

(**A**) Representative Western blot showing the different Aβ species present in the oligomeric preparation described in the Materials and Methods section. (**B**) Treatment of hippocampal neurons for 24 hours with 10 µM of oligomeric Aβ resulted in a significant amount of cell death compared to control cells, treated with fresh Neurobasal medium. Cell death was quantified from at least 3 independent experiments and expressed as the mean+/-SEM (*p=0.012) number of viable cells per field. (**C**) Representative image showing significant neuritic dystrophy in neurons treated with 10 µM Aβ for 24 hours.

Figure 4-4. Effects of oligomeric Aβ on dendritic spines

Treatment of hippocampal neurons for 24 hours with a 300 nM solution of oligomeric Aβ resulted in a significant decrease in dendritic spine density, compared to control cells that were treated with fresh Neurobasal medium. Spine effects were measured by direct count of dendritic spines (**A**, **B**) and by measuring the levels of two dendritic spine markers, PSD95 (**C**, **D**) and Drebrin A (**E**, **F**). (**A**) Dendritic spines were quantified from an average of 12 neurons from at least 3 independent experiments, and expressed as the mean+/-SEM (*p=0.023) number of spines/ μM. (**B**) Representative image showing a decrease in dendritic spines in Aβ-treated neurons compared to control neurons. (**C**, **E**) Representative Western blots showing a decrease in the synaptic markers PSD-95 and Drebrin A in Aβ-treated neurons. (**D, F**) Densitometric analysis of data was quantified from 3 independent experiments and expressed as the mean+/-SEM (*p=0.034 for PSD95; *p=0.013 for Drebrin A) band intensity.

The exact mechanism(s) by which Aβ initiates these effects remains unclear. Several lines of evidence have shown that dimerization of APP by Aβ may play a key role in Aβ-induced toxicity (Lorenzo et al., 2000; Lu et al., 2003a; Shaked et al., 2006). We wondered whether the observed toxic effects were intrinsic properties of Aβ, or whether signaling through APP alone was a sufficient trigger. To address this question we tested

whether oligomerization of APP by 22C11 could recapitulate the neurotoxic effects of oligomeric Aβ. Previous reports have demonstrated that 22C11 is highly toxic to cultured cortical neurons (Rohn et al., 2000). Nevertheless, we had two objectives. First, we wanted to confirm the neurotoxic effects of 22C11 on cultured hippocampal neurons. Second, we set out to establish a dose-response curve to identify a concentration at which the neurotoxic effects of 22C11 were minimized while still triggering synaptic alterations in these neurons. Synaptic dysfunction is thought to be one of the earlier events in the progression of AD, with neuronal death occurring at a later stage (Delaere et al., 1989; Terry et al., 1991). Our results are presented below.

4.2.3 Hippocampal neurons treated with 22C11 undergo apoptosis

To establish a dose-response curve for the neurotoxic effects of 22C11, we treated cultured hippocampal neurons with various concentrations of 22C11 (100 pg/ml, 100 ng/ml and 1.0 µg/ml) for 24 hours. A count for cell viability showed that 22C11 triggered neuronal death (~20%) starting at a concentration of 1.0 µg/ml. Lower concentrations of 22C11 (i.e. 100 pg/ml and 100 ng/ml) did significantly not affect neuronal viability after 24 hours (Figure 4-5A). The possibility remains however that these lower concentrations of 22C11 could be toxic after a longer exposure. As was previously reported (Rohn et al., 2000), we observed a significant amount of neuritic dystrophy in neurons exposed to toxic concentrations of 22C11 (Figure 4-5B), which was not apparent at the sub-apoptotic concentrations (data not shown).
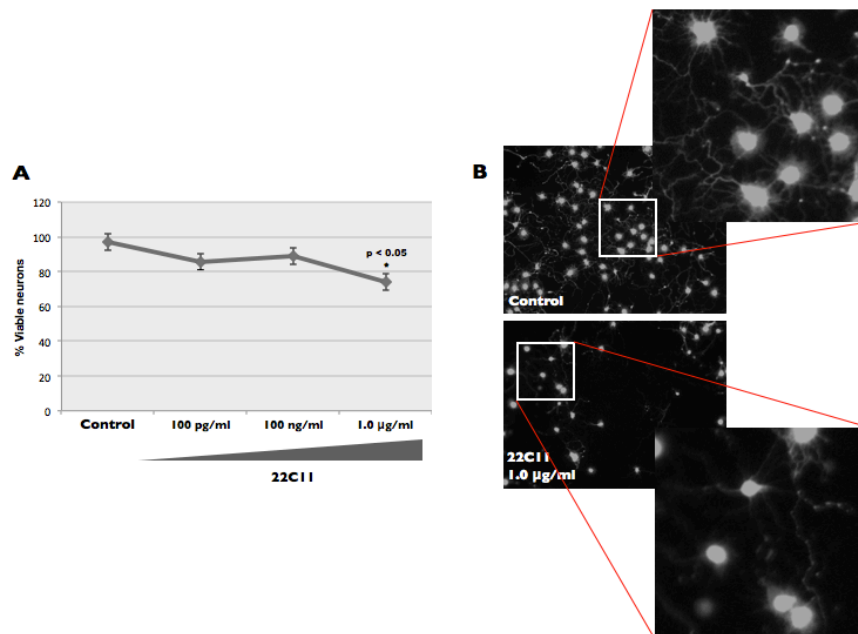
Figure 4-5. Neurotoxic effects of 22C11 in hippocampal neurons

(**A**) Dose-response curve for hippocampal neurons over 24 hours with various concentrations of 22C11 showed a significant amount of cell death at 1.0 µg.ml compared to control cells, which were treated with PBS. Lower concentrations of 100 pg/ml and 100 ng/ml had no significant neurotoxic effects after 24 hours. Cell death was assessed with the LIVD/DEAD cell viability assay kit and quantified the from at least 3 independent experiments and expressed as the mean+/-SEM (*$p<0.05$) number of viable cells per field. (**B**) Representative image showing a significant amount of neuritic dystrophy in cells treated with 1.0 µg/ml of 22C11 for 24 hours.

4.2.4   22C11-induced cell death is dependent on APP

To confirm that the effects of 22C11 were triggered by binding APP, we treated a murine neuroblastoma cell line B103, previously characterized (Schubert and Behl, 1993) as being devoid of APP and APLPs, with 22C11 (5.0 µg) for 24 hours. We found that these cells were completely resistant to the toxic effects of 22C11 (Figure 4-6A). One the other hand, B103 cells that stably expressed APP were found to be vulnerable to the effects of 22C11 after 24 hours (Figure 4-6A), confirming that APP is required for the

toxic effects of 22C11. These results suggest that 22C11 induces its toxic effects by specifically interacting with APP at the cell surface. We did not test whether APLP1 or APLP2 could restore the neurotoxic effects of 22C11 on B103 cells. While 22C11 is highly specific for APP, it has been shown to cross-react, albeit weakly with both APLP1 and APLP2 (Wang et al., 2006). Therefore, it is possible that they may contribute as well to the toxic effects of 22C11 in neurons, which express APLPs.

4.2.5   22C11-induced toxicity is dependent on its ability to dimerize cell surface APP

While the experiments described above suggest that APP is required for the toxic effects of 22C11, they do not address whether oligomerization of APP is also required. To tackle this question, we treated hippocampal neurons with isolated monovalent Fab fragments of 22C11 (5.0 µg/ml), which retain the ability to bind cell surface APP without being able to dimerize it. As expected, 22C11 Fab fragments had almost no effects on the treated neurons after 24 hours (Figure 4-6B), consistent with the idea that APP dimerization is essential for triggering the signaling pathway leading to observed toxicity.
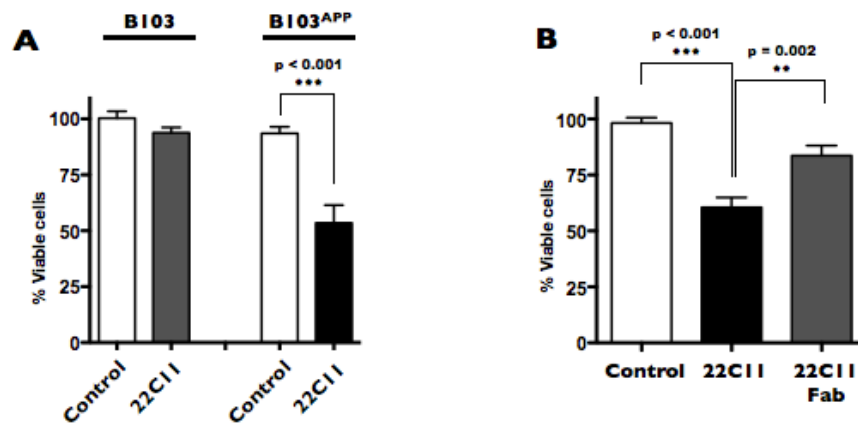
Figure 4-6. Neurotoxic effects of 22C11 require APP and are dependent on its ability to dimerize surface APP

(**A**) Treatment of B103 cells with a 5 µg/ml solution of 22C11 had no significant neurotoxic effects compared to control cells treated with PBS. Alternatively, similar treatment of B103 stably expressing APP resulted in a significant decreased in cell viability when compared to control cells treated with either PBS. Cell death was quantified from at least 3 independent experiments and expressed as the mean+/-SEM (***$p < 0.001$ for B103APP cells) number of viable cells per field. (**B**) Treatment of hippocampal neurons with 2.5 µg of Fab fragments of 22C11 did show any significant toxicity compared to the same concentration of the intact antibody. Cell death was quantified from at least 3 independent experiments and expressed as the mean+/-SEM (***$p < 0.001$ for 22C11; **$p = 0.002$ for 22C11-Fab) number of viable cells per field.

4.2.6 Sub-apoptotic concentrations of 22C11 induce synaptic dysfunction in hippocampal neurons

Having confirmed that 22C11 is toxic to hippocampal neurons and having established a dose-response curve for it, we next set out to investigate whether a low concentration of 22C11 could mimic the synaptotoxic effects of sub-lethal levels of oligomeric Aβ_{42}. To investigate this, hippocampal neurons were treated with a sub-apoptotic concentration of 22C11 (100 ng/ml) for 24 hours. A direct count of the number of dendritic spines revealed a significant decrease in their density, by as much as ~40%, in neurons treated with 22C11 compared to control neurons treated with PBS (Figure

4-7A, B). As was the case with Aβ (300 nM), the observed decline in overall spine numbers was accompanied by a corresponding reduction in the protein levels of the dendritic spine markers PSD-95 (Figure 4-7C, D) and Drebrin A (Figure 4-7E, F) after the 24 hour period.

Figure 4-7. Effects of 22C11 on dendritic spines in hippocampal neurons

Treatment of hippocampal neurons for 24 hours with a 100 ng/ml solution of 22C11 resulted in a significant decrease in dendritic spine density, compared to control cells that were treated with PBS. Spine effects were measured by direct count of dendritic spines (**A**, **B**) and by measuring the levels of two dendritic spine markers, PSD95 (**C**, **D**) and Drebrin A (**E**, **F**). (**B**) Dendritic spines were quantified from an average of 12 neurons taken from at least 3 independent experiments, and expressed as the mean+/-SEM (**p=0.009) number of spines/μM. (**C**, **E**) Representative Western blots showing a decrease in the synaptic markers, PSD95 and Drebrin A in 22C11-treated neurons. (**D**, **F**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (**p=0.005 for PSD95; ***p<0.001 for Drebrin A) band intensity.

4.2.7 22C11-induced loss of dendritic spines is dependent on oligomerization of cell-surface APP

The fact that the monovalent Fab fragments of 22C11 had no demonstrable toxicity, suggests that dimerization of APP is necessary for the neurotoxic effects of 22C11. We set out to evaluate whether the effects of 22C11 on synaptic function were also dependent on the dimerization of APP. Again, hippocampal neurons were treated with the isolated Fab fragments of 22C11 (100 ng/ml) for 24 hours. As expected, we found no significant changes in dendritic spine density in neurons treated with the Fab fragments, whereas intact 22C11 reduced dendritic spine density by ~50%. Control cell treated with PBS had no adverse effects (Figure 4-8).

Figure 4-8. The effects of 22C11 on dendritic spines in hippocampal neurons are dependent on its ability to oligomerize cell-surface APP
Treatment of hippocampal neurons for 24 hours with the Fab fragment of 22C11 (100 ng/ml) showed no significant effects in dendritic spine density compared to the same concentration of the intact antibody. Dendritic spines were quantified from an average of 12 neurons taken from at least 3 independent experiments, and expressed as the mean+/-SEM (***p<0.001) number of spines/μM.

4.3   Discussion

Much has been learned about APP since its discovery over 20 years ago (Kang et al., 1987). However, many more questions have remained unanswered, particularly with regard to the role of the intact, full-length form of APP in AD pathology and in neuronal

plasticity. In fact, while the vast majority of synthesized APP is metabolized in various cell compartments, with the fragments being secreted or degraded (reviewed by Chow et al., 2009), a small proportion of transmembrane APP remains stably localized to the plasma membrane (Storey et al., 1999). Different lines of evidence have suggested a growth promoting (references) or cell-adhesion promoting (references) role for transmembrane APP due to its propensity to dimerize, reminiscent of tyrosine kinase receptors. This has led many to wonder whether perturbation of the APP monomer/ dimer equilibrium could play a role in AD. To investigate this problem, we employed an artificial system, making use of the divalent mouse monoclonal antibody (clone 22C11) to dimerize APP. This technique had previously been utilized to study similar effects in cortical neurons (Mbebi et al., 2002; Rohn et al., 2000); however in our studies we focused primarily on hippocampal neurons, since the hippocampus is one of the region that is most affected in AD (reviewed by Burger, 2010).

The usage of an antibody can be used to facilitate oligomerization of surface APP. Interestingly, similar to other published data (Gralle et al., 2009; Scheuermann et al., 2001), we found that a large proportion of cell-surface APP could be isolated as dimers and tetramers under normal conditions, albeit in APP over-expressing cells. These APP complexes were isolated in larger quantities (especially tetrameric APP) in cells that had been treated with the antibody 22C11, suggesting that the antibody acts to stabilize preformed cell-surface APP oligomers, rather than inducing oligomerization *per se* of APP. Based on our results and other published data, we concluded that 22C11 is a viable method for artificially shifting the APP monomer/multimer equilibrium towards the higher APP aggregates.

Additionally, our results demonstrate that 22C11 can induce apoptosis in hippocampal neurons, as well as the previously reported cortical neurons (Rohn et al., 2000),

suggesting that shifting the APP oligomerization equilibrium was sufficient to activate one or more pathways leading to neuronal death. In contrast, we found no neurotoxic effects in neurons treated with the isolated Fab fragment of 22C11, supporting our hypothesis that oligomerization of APP by 22C11 is necessary for the observed neurotoxicity. Complementary evidence corroborating this model came from the work of Gralle *et al.*, who showed that the neuroprotective effects of sAPPα were due to its ability to destabilize APP dimers (Gralle et al., 2009). Moreover, a significant amount of neuritic dystrophy (not quantified) was observed in the treated neurons, which was apparent as early as 8 hours, when the majority of neurons appeared to be otherwise viable, suggesting that neurite degeneration precedes apoptosis. Whether this accompanying neuritic dystrophy is a result of a process affecting the whole cell or due to 22C11 inducing a local, deleterious effect on neuritic processes remains unclear.

We found that these effects required the presence of APP, as B103 cells lacking APP were resistant to the effects of 22C11. Because these cells also lack APLP1 and APLP2, we cannot rule out the possibility that they also contribute to the neurotoxic effects of 22C11 in our model. Indeed, most of the functional domains and structural motifs found in APP are also present in APLPs, particularly APLP2 (White et al., 1998), suggesting that they may share common biological functions in neurons. In support of this possibility, cortical neurons taken from APP-knockout mice were shown to retain their vulnerability in an antibody-mediated death model (Mbebi et al., 2002) and the fact that 22C11 can cross-react weakly with APLP2 (Wang et al., 2006). However, since B103 cells lack APLP2, our studies demonstrate that APP alone is sufficient for the neurotoxic effects of 22C11.

Finally, while neuronal death is a common occurrence in AD, there is ample evidence suggesting that synaptic dysfunction correlates very well with memory impairments and

may precede not only amyloid plaques, but neuronal apoptosis as well in mouse models of AD (Chapman et al., 1999; Cleary et al., 2005; Hsia et al., 1999; Lesné et al., 2006; Rowan et al., 2007; Selkoe, 2002). Synaptic transmission and plasticity were shown to be altered in neurons over-expressing APP (Kamenetz et al., 2003) or exposed to elevated levels of Aβ (Freir et al., 2001; Hartley et al., 1999; Vitolo et al., 2002), which also resulted in a significant decrease in dendritic spine numbers (Shrestha et al., 2006). Interestingly, both of these paradigms lead to an increase in dimerized APP (Lu et al., 2003a; Scheuermann et al., 2001; Shaked et al., 2006), which led us to examine the effects of sub-apoptotic concentrations of 22C11 on synaptic functions in hippocampal neurons. Our results showed a significant reduction in dendritic spine numbers, as well as a decrease in the synaptic markers PSD-95 and Drebrin A in 22C11-treated neurons, suggesting that increased dimerization of APP can exert localized effects on synaptic function without affecting neuronal viability. Whether or not apoptotic and sub-apoptotic concentrations of 22C11 modulate the same signaling pathway remains uncertain. It is possible that 22C11 affects synaptic contacts by shifting the APP dimer equilibrium from an intercellular trans-configuration to a intracellular cis-configuration. The evidence suggests that increased dimerization of APP may affect one or more pathways, perhaps through caspase-3 activation (Rohn et al., 2000) or by disrupting Ca^{2+} entry in the cells (Bouron et al., 2004) or by stimulating pro-inflammatory cytokine release (Sondag and Combs, 2004; Sondag and Combs, 2006) or perhaps by simulating the production and release of Aβ (Kaden et al., 2008; Kaden et al., 2009; Kienlen-Campard et al., 2008; Munter et al., 2010; Munter et al., 2007; Scheuermann et al., 2001; Sondag and Combs, 2006). In the following chapter, we will explore the latter hypothesis that dimerization of APP regulates the production of Aβ in hippocampal neurons.

CHAPTER 5: ABERRANT DIMERIZATION OF APP TRIGGERS THE AMYLOIDOGENIC PATHWAY AND INCREASES Aβ LEVELS IN HIPPOCAMPAL NEURONS

5.1   Introduction

The progressive accumulation and deposition of the β-amyloid peptide (Aβ) leading to the formation of senile plaques (SP) is an invariant feature of Alzheimer's disease. The amyloidogenic processing of the amyloid precursor protein (APP; Kang et al., 1987) by the β- and γ-secretases leading to the production of Aβ is a well characterized process (reviewed by Walsh et al., 2007). APP and APLPs were conventionally thought to exist and function as monomers. However, biochemical and structural data obtained over the past years have indicated that APP and APLPs may in fact exist as functional dimers or even present in higher oligomeric complexes (Chen et al., 2006; Gralle et al., 2009; Kaden et al., 2008; Rossjohn et al., 1999; Scheuermann et al., 2001; Wang and Ha, 2004). Many studies have implicated the APP and APLP homo- and heterotypic interactions in cellular adhesion (Soba et al., 2005). Among other proposed mechanisms, the strength and degree of APP dimerization has been reported to influence APP processing (Kaden et al., 2008; Scheuermann et al., 2001) and many have suggested that aberrant dimerization of APP may play a critical factor in the pathogenesis of late-onset Alzheimer's disease (LOAD). The identification of hereditary early-onset familial Alzheimer's disease (EOFAD) mutations in PS1 and particularly in APP have provided ample support for this view.

Most EOFAD mutations are known to increase Aβ. It has been proposed that this increase may be driven by abnormal dimerization of APP through its transmembrane

sequence (TMS). APP contains three GxxxG motifs at the junction between the juxtamembrane and the TMS regions (Liu et al., 2005; Marchesi, 2005; Munter et al., 2007; Sato et al., 2006). GxxxG motifs were originally identified in the sequence of the glycophorin A (GpA) protein where they mediate sequence-specific dimerization between transmembrane helices by direct glycine-glycine contacts (Bormann et al., 1989; Lemmon et al., 1994; Smith et al., 2001). Since their discovery, GxxxG motifs have been shown to play a generic role to facilitate oligomerization of TM domains of many transmembrane proteins (Russ and Engelman, 2000), including APP. The presence of these three GxxxG motifs have led to the suggestions that the glycine face of the TMS helix of APP may mediate APP homo- and hetero-oligomerization (Kienlen-Campard et al., 2008; Munter et al., 2007). Interestingly, one of the identified mutations in EOFAD, the Flemish mutations, involves the substitution of an alanine residue to a glycine residue resulting in the creation of a fourth GxxxG motif in APP (Hendriks et al., 1992). Moreover, mutations of the glycine residues of the GxxxG motifs to alanine residues attenuate TM dimerization and result in a marked reduction in the processing of APP and the production of Aβ (Munter et al., 2010). Similarly, compounds that directly interfere with APP TMS dimerization were recently demonstrated to lower Aβ levels (Richter et al., 2010).

The evidence presented above supports the view that APP dimerization plays a role in its processing and enhances Aβ production. However, the precise mechanism(s) by which homo-dimerization of APP affects its proteolytic cleavage remains largely unresolved. In this study we show that enhanced dimerization of APP triggers amyloidogenic processing of APP in hippocampal neurons, resulting an increased Aβ production. Moreover, we demonstrate that increased Aβ production is due to an increase in the protein levels and activity of the β-secretase BACE1. We demonstrate

that elevated levels of BACE1 stem from decreased lysosomal degradation through a caspase-3 dependent mechanism, leading to an accumulation of BACE1 in endosomal compartments.

5.2   Results

5.2.1 22C11 treatment increases Aβ levels in cultured hippocampal neurons without affecting cell viability

To investigate whether dimerization of APP played a direct role in increasing the levels of Aβ in neurons, cultured hippocampal neurons were treated with a sub-lethal concentration of the N-terminal APP antibody 22C11 (100 ng/ml; Figure 4-7) as previously described. After treatment, cells were harvested and the levels of intraneuronal Aβ were measured using two independent techniques. First, Aβ was immunoprecipitated from the neuronal lysates with the monoclonal anti-Aβ antibody 4G8. We detected a significant increase in Aβ production (~2-fold) in 22C11-treated neurons compared PBS-treated control neurons (Figure 5-1A). Additionally, we did not detect any significant changes in the levels of full-length APP either in 22C11- nor PBS-treated neurons (Figure 5-1), suggesting that 22C11 treatment increases Aβ levels by directly affecting its production or clearance.

In a second approach, the levels of Aβ in the lysates were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, which measures the levels of $A\beta_{(x-42)}$. Consistent with our immunoprecipitation results, we found a significant increase in the levels of intraneuronal $A\beta_{(x-42)}$ in the 22C11-treated cells compared to PBS-treated control cells (Figure 5-1B). Interestingly, we did not

detect a significant change in the levels of secreted Aβ in the culture medium of either 22C11-treated neurons nor PBS-treated control cells, as measured by ELISA or immunoprecipitation (data not shown).

Figure 5-1. 22C11 treatment increases Aβ levels in hippocampal neurons

Treatment of hippocampal neurons for 24 hours with a 100 ng/ml solution of 22C11 resulted in a significant increase in Aβ production compared to control cells that were treated with PBS. Aβ levels were assessed by immunoprecipitation with the anti-Aβ antibody 4G8, from cell lysates (**A**) and by measuring secreted Aβ in the culture medium by ELISA (**C**). (**A**) Representative Western blot showing an increase in Aβ levels in 22C11-treated neurons. Data was quantified from at least 3 independent experiments and expressed as the mean+/-SEM (**p=0.039) band intensity. (**B**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (**p=0.005 for PSD95; ***p<0.001 for Drebrin A) band intensity. (**C**) Quantification of Aβ$_{(x-42)}$ from culture medium by ELISA from 4 independent experiments was expressed as the mean+/-SEM (*p=0.033) signal intensity in arbitrary units (AU).

5.2.2 Increased Aβ levels by 22C11 correlates with increased BACE1 levels and activity but not with increased γ-secretase activity

Our next objective was to determine the mechanism by which Aβ levels were increased in 22C11-treated neurons. Since the levels of APP remained unchanged in the treated cells, we hypothesized that the changes in Aβ levels might be due to a perturbation in the equilibrium between its clearance and production rates. As we discussed earlier

(Section 1.9.2), several studies have implicated the metalloproteases neprilysin and IDE in the degradation of Aβ (Figure 5-2A; reviewed in Miners et al., 2008). The levels of those two proteins have also been shown to be reduced in several AD mouse models and in AD patients (Apelt et al., 2003; Cook et al., 2003; Kurochkin and Goto, 1994; Yasojima et al., 2001). To test whether a deficiency in clearance correlated with the increased Aβ levels in our system, we measured the protein levels of IDE and neprilysin by Western blot, using commercially available polyclonal antibodies. We did not detect any significant changes in the protein levels of either IDE nor neprilysin (Figure 5-2B) in the 22C11-treated neurons after 8 hours, indicating that increased β- and/or γ-secretase processing of APP (Figure 5-2B) was the likely cause of the elevated Aβ levels.

Figure 5-2. 22C11 does not affect Aβ degradation in neurons

(**A**) Aβ is degraded by insulin degrading enzyme (IDE) and Neprilysin (Farris et al., 2007; Selkoe, 2001). (**B**) Western blot analysis of lysates from neurons treated with 100 ng/ml of 22C11 for 8 hours did not show any significantly changes in the protein levels IDE or Neprilysin compared to control neurons treated with vehicle.
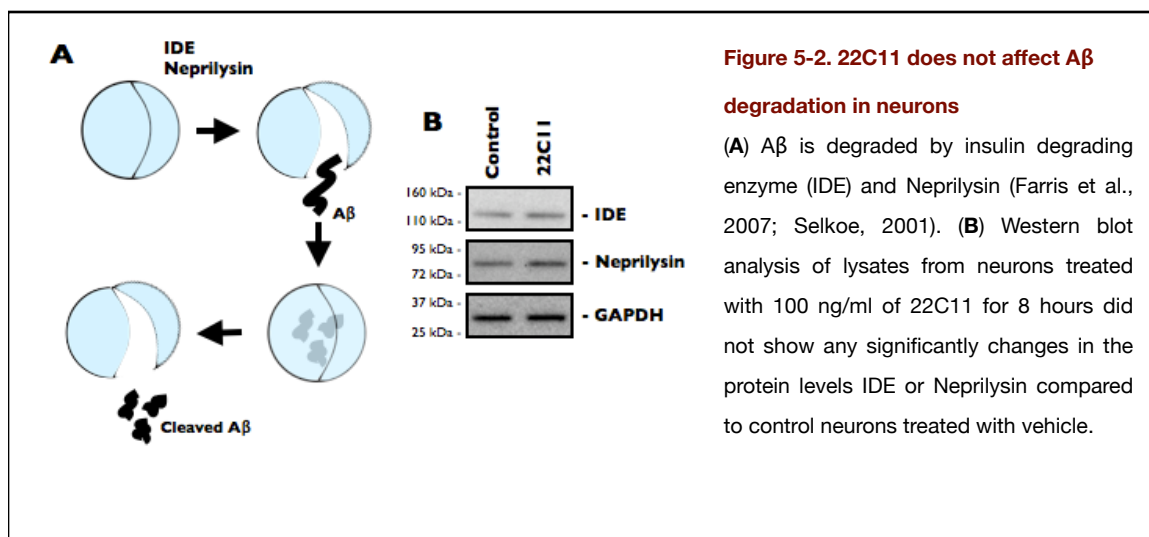
To evaluate γ-secretase processing of APP, we used two different approaches. Active endogenous presenilin (PS) exists mainly as a heterodimeric complex formed from the endoproteolytically processed N- and C-terminal fragments of PS (Figure 5-3A; Thinakaran et al., 1996a). Therefore, we measured the levels of N-terminal fragments (~28 kDa) by Western blot, using a commercially available N-terminal antibody for presenilin-1. There was no statistical difference in the levels of cleaved PS1 N-terminal

fragments between neurons that were treated with 22C11 and vehicle (PBS)-treated control neurons (Figure 5-3B). As a potential read-out of γ-secretase activity, we evaluated cleavage of the γ-secretase substrate Notch1 (Ray et al., 1999; Steiner et al., 1999), by Western blot using a polyclonal antibody which recognizes endogenous levels of the cytosolic domain of Notch1 only when cleaved between Glycine^{1743} and Valine^{1744} (Ray et al., 1999). Interestingly, we detected a modest, albeit statistically insignificant increase in Notch1 cleavage (Figure 5-3B) in 22C11 treated neurons.

Figure 5-3. 22C11 treatment does not affect cleavage of presenilin-1 protein

(**A**) Topology and proteolytic processing of presenilins. Presenilins are cleaved within the hydrophobic region of the large cytosolic loop between TM6 and TM7, resulting in the formation of a heterodimeric complex composed of the N-terminal fragment (NTF) and the C-terminal fragment (CTF). (**B**) Western blot analysis of lysates prepared from hippocampal neurons treated with 22C11 (100 ng/ml) for 8 hours. No significant increases in the levels of N-terminal fragments of presenilin-1 nor in the levels of the γ-secretase substrate Notch were observed in treated neurons compared to PBS-treated control.

Because of the modest increase in Notch1 cleavage in 22C11-treated cells, we could not rule out the possibility that γ-secretase activity was enhanced by 22C11 through a mechanism distinct from increasing PS1 cleavage. Therefore, we decided to directly measure cleavage of CTFβ (C99) by γ-secretase using a previously described cell-free assay (Hansson et al., 2006; Sastre et al., 2001) as depicted in Figure 5-4A. Briefly, HEK293 cells were transiently transfected with a C99-myc construct and membrane

fractions were prepared from these cells 48 hours post-transfection. These membrane fractions, enriched with APP C99-myc fragments (Figure 5-S3), were combined with solubilized hippocampal neurons (γ-secretase complex source) from 22C11-treated and PBS-treated cells and incubated for 2 hours at 37°C to allow γ-secretase cleavage of C99-myc. The generated AICD-myc fragments were then analyzed by Western blot using a polyclonal anti-myc antibody and were detected in both control and 22C11-treated samples (Figure 5-4B. We did not observe a significant difference across the samples (Figure 5-4C). γ-Secretase-mediated cleavage of C99-myc, was confirmed by the addition of the γ-secretase inhibitor DAPT (Dovey et al., 2001), which completely abolished generation of AICD-myc fragments (Figure 5-4B).

Figure 5-4. 22C11 treatment does not increase γ-secretase activity

(**A**) Complexed N- and C-terminal fragments of cleaved presenilin-1 (Psn-1) are stabilized by binding to Aph-1 and nicastrin, although the complex still remains inactive. Binding of Pen-2 elicits the final step of maturation of the γ-secretase complex, facilitating endoproteolysis of PS1 and conderring γ-secretase activity. (**B, C**) *In vitro* γ-secretase activity assay with hippocampal neurons treated with 22C11 for 8 hours. Solubilized membranes from C100-myc expressing HEK293 cells were mixed with solubilized 22C11-treated (100 ng/ml; 8 hours) or vehicle-treated (PBS) hippocampal neurons. Samples were incubated for 2 hours in the absence or presence of the γ-secretase inhibitor (DAPT). (**B**) AICD-myc formation at 37°C was detected by Western blot with a myc-specific antibody. No AICD was formed at -20°C or in the presence of DAPT at 37°C. (**C**) No observable increase in AICD production was observed in 22C11-treated compared to vehicle-treated neurons. Quantification of AICD-myc from 3 independent experiments, expressed as the relative mean+/-SEM (p=0.231) signal intensity.

To assess β-secretase cleavage of APP, we first measured the total levels of cellular BACE1 protein in hippocampal neurons following an 8-hour treatment with a sub-lethal concentration of 22C11. BACE1 levels were determined by Western blot using a commercially available polyclonal anti-BACE1 antibody (Figure 5-5B). We detected a significant increase in total BACE1 protein levels, normalized against GAPDH, in 22C11-treated neurons compared to PBS-treated control cells (Figure 5-2C). To correlate increased BACE1 levels with increased activity, we measured the levels of sAPPβ (Figure 5-5A) in the culture medium by Western blot, using a commercially available monoclonal sAPPβ-specific antibody (Figure 5-5B). Concomitant with the increase in BACE1, sAPPβ levels were significantly higher in the culture medium from 22C11-treated neurons compared to that of control cells (Figure 5-2C), consistent with the idea that increased BACE1 protein leads to increased β-secretase processing of APP.

Figure 5-5. BACE1 protein level and activity are increased in 22C11-treated hippocampal neurons

(**A**) Schematic illustration of of APP by BACE1, generating the soluble sAPPβ fragment as well as the membrane bound C100 fragment. (**B-D**) Treatment of primary hippocampal neurons with 22C11 (100 ng/ml) for 8 hours resulted in Increased BACE1 protein levels with a concomitant increase in secreted APPβ fragments as measured by (**B**) Western blot analysis. (**C, D**) Densitometric analysis of the data was quantified at least 3 independent experiments and expressed as the mean+/-SEM (***$p<0.001$ for BACE1; *$p=0.05$ for sAPPβ) band intensity.

Taken together, these results suggest that the observed increase in the levels of intracellular Aβ in 22C11-treated hippocampal neurons, is unlikely caused by a deficiency in the clearance mechanism of Aβ, supported by the unchanged levels of IDE and nepriplysin in 22C11-treated neurons. Instead, our results led us to propose that increased processing of APP, specifically by BACE1 at the β-secretase cleavage site of APP was responsible for driving the elevated Aβ levels.

5.2.3 Increases in BACE1 protein levels are not caused by increased transcription or translation but are due to increases in protein stability

Having demonstrated that levels of BACE1 protein were elevated in 22C11-treated hippocampal neurons, our next objective was to investigate potential mechanisms to explain this observation. Several factors might be in play, including transcriptional, post-transcriptional, translational and/or post-translational modifications (reviewed in Tabaton et al., 2010).

We first investigated the possibility that 22C11 could activate transcription of BACE1 in hippocampal neurons. qRT-PCR analysis of BACE1 mRNA levels in 22C11 treated-neurons did not reveal any significant changes over control neurons after 8 hours (Figure 5-6A). Since BACE1 protein levels was already shown to be elevated at this time point (Figure 5-5B, C), we ruled out the possibility that 22C11 could affect transcriptional control of BACE1. This is in line with several findings that have failed to show a significant increase in BACE1 mRNA levels in AD brains and in AD mouse models, in spite of elevated BACE1 protein levels (Hébert et al., 2008; Holsinger et al., 2002; Zhao et al., 2007).

An alternative explanation for the increase in BACE1 protein levels is the perturbation of one or more of its post-transcriptional regulatory mechanisms. Regulatory noncoding RNAs (ncRNA) are widely recognized today for their potent roles in regulating gene expression in many key developmental processes (reviewed by Brosnan and Voinnet, 2009). The neuronal functions of ncRNAs have only begun to be explored and are for the most part unknown. However, specific ncRNAs have been shown to regulate dendritic spine development, neuronal fate specification and differentiation and synaptic protein synthesis (reviewed in Satterlee et al., 2007). Additionally, ncRNAs have been implicated in a number of neuronal diseases, including Tourette's syndrome and Fragile X syndrome (reviews by Iacoangeli et al., 2010; Mehler and Mattick, 2007). Recently, a potential role for ncRNAs was proposed in AD with two the discovery of several novel ncRNAs, namely a naturally occurring BACE1-antisense transcript (BACE1-AS; Faghihi et al., 2008) and its binding competitor (miR-485-5p; Faghihi et al., 2010), and a microRNA cluster (miR-29a/b-1; Hébert et al., 2008). They have all been implicated in regulating the stability of BACE1 mRNA and their misexpression have been shown to correlate with increased Aβ production.

To test the hypothesis that BACE1 was elevated due to increased synthesis from stabler mRNAs, we performed a cycloheximide degradation assay to directly study changes in the turnover rate of BACE1. Hippocampal neurons were treated with cycloheximide to prohibit protein synthesis and BACE1 protein levels were evaluated by Western blot over the course of 18 hours. At that time point, BACE1 protein levels were almost completely depleted in control cells (Figure 5-6B). Alternatively, in 22C11-treated neurons, BACE1 protein levels were elevated after 3 hours and fell only to control levels over the course of 18 hours (Figure 5-6B). Comparing neurons at the 18 hour mark showed a significantly higher amount of endogenous BACE1 protein in 22C11-treated neurons (Figure 5-6C). This suggests that 22C11 affects the turnover rate of BACE1,

increasing the half-life of endogenous BACE1 protein. These results rules out increased translation as the cause of elevated BACE1 levels.

Figure 5-6. 22C11-treatment leads to accumulation of BACE1 through post-translational stabilization, not through increased synthesis

(**A**) qRT-PCR assay for BACE1 mRNA: primary hippocampal neurons were treated with 22C11 (100 ng/ml) for 8 hours prior to analysis. No significant changes in BACE1 mRNA levels were observed between 22C11- and vehicle (PBS)-treated neurons. The data was quantified from 3 independent experiments and is expressed as the relative mean+/-SEM (p=0.193) signal intensity. (**B**, **C**) Cycloheximide (CHX) degradation time course: BACE1 protein was detected by Western blot at various times (3, 6 and 18 hours) after addition of CHX (150 µM) only or 22C11 (100 ng/ml) + CHX in hippocampal neurons. The degradation of BACE1 in control neurons after 18 hours was blocked by the addition of 22C11.

Collectively, the above data suggests that 22C11 drives Aβ production by altering the steady-state levels of BACE1, the rate-limiting enzyme in the biogenesis of Aβ. They also indicate that the increase in BACE1 levels is, at least in part, the result of post-transcriptional stabilization of BACE1.

5.2.4 The lysosomal pathway is the predominant route for degradation of endogenous BACE1 in hippocampal neurons

At least three different mechanisms have been reported to control the degradation of BACE1: (1) endoproteolysis within its catalytic domain (Huse et al., 2003), (2) the lysosomal pathway (Koh et al., 2005), and (3) the ubiquitin-proteasomal pathway (Qing et al., 2004). The discrepancy between the last two studies is puzzling, but can be reconciled. The latter study by Qing *et al.* was largely based on over-expression of BACE1 in several cell lines. Hence, it remains unclear whether endogenous BACE1 would normally be degraded through the proteasomal pathway. In addition, since the effects of lysosomal inhibitors were not studied, any potential contribution from lysosomal degradation to the degradation of BACE1 is unknown. Moreover, the interpretation of experiments involving proteasome inhibitors can be problematic since many proteasome inhibitors can also inhibit lysosomal cathepsins (Kisselev and Goldberg, 2001; Kozlowski et al., 2001).

In order to overcome these shortcomings, we decided to evaluate the respective contributions of proteasomal and lysosomal degradative pathways in regulating the levels of endogenous BACE1 in our model system, namely hippocampal neurons. To assess lysosomal degradation of endogenous BACE1, hippocampal neurons were treated for 8 hours with ammonium chloride (NH_4Cl; 500 µM) and chloroquine (100 µM), weak bases known to inhibit lysosomal hydrolases by reducing the acidification of the endosomal/lysosomal compartments (Ohkuma and Poole, 1978). After treatments, neurons were harvested and the levels of BACE1 protein were evaluated by Western blot analysis (Figure 5-7A). Both chloroquine and NH_4Cl treatments induced a marked increase in endogenous BACE1 protein levels after 8 hours (Figure 5-7B). Increasing the concentrations each inhibitor did not appear to significantly affect the build-up of

BACE1. However, longer treatments did lead to higher BACE1 levels (data not shown). These results are in line with previous studies that demonstrated a similar effect in several cell lines, including CHO and SY5Y cells and in cortical neurons (Koh et al., 2005). To assess proteasomal degradation, a similar protocol was followed and cultured hippocampal neurons were treated with the proteasomal inhibitor MG132 (1.0 µM) for 8 hours. Western blot analysis (Figure 5-7C) showed no significant increase in BACE1 levels after 8 hours (Figure 5-7D). Increasing the concentration of MG132 or extending the treatment over a longer time frame proved problematic due to toxicity. Comparable results were also obtained with another proteasomal inhibitor, lactacystin (data not shown).

Figure 5-7. Inhibition of lysosomal hydrolase leads to increased levels of endogenous BACE1 in hippocampal neurons

Primary hippocampal neurons were treated with various protease inhibitors for 8 hours (**A**-**D**). (**A**) Western blot analysis of lysates prepared from neurons treated with the lysosomal inhibitors Chloroquine (100 µM) and NH4Cl (500 µM) resulted in a significant increase in BACE1 levels with a concomitant increase in the levels of the C100 fragment resulting from BACE1 cleavage of APP. (**B**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (**p=0.002 for chloroquine; **p=0.004 for NH4Cl) band intensity. (**C**, **D**) Western blot analysis of lysates from neurons treated with the proteasomal inhibitor MG132 (1.0 µM) did not significantly affect BACE1 protein levels after 8 hours. (**D**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (p=0.099) band intensity.

These results, taken collectively, indicate that endogenous BACE1 degradation in hippocampal neurons is primarily determined by the activity of lysosomal hydrolases.

Any contribution from proteasomal degradation appears to be minimal, at least within the time frame of our experiments.

5.2.5 22C11 treatment interferes with the sorting properties of BACE1 by GGA3

The dileucine (DXXLL) motif of BACE1 (discussed in Section 1.3.2) is believed to play a critical role in its degradation, as mutating the leucine residues to alanine residues increases the levels of BACE1 protein (Pastorino et al., 2002). Mutagenesis of the dileucine motif has been shown to interfere with the lysosomal degradation of BACE1 by blocking its transport from endosomes to lysosomes (Koh et al., 2005). This trafficking pathway is mediated by the Vps-27, Hrs and STAM (VHS) domain of the Golgi-localized gamma-ear-containing ARF-binding (GGA) proteins (GGA1, 2, and 3), which binds the dileucine motif of BACE1 (reviewed in Bonifacino, 2004). Depletion of GGA proteins by RNAi increases accumulation of BACE1 in early endosomes (He et al., 2005; Tesco et al., 2007; Wahle et al., 2005) and increases Aβ secretion in neurons (Wahle et al., 2006). Alternatively, over-expression of GGA proteins or dominant-negative variants reduce CTFβ generation and Aβ production (Wahle et al., 2006). Interestingly, of the three GGA family member proteins, GGA3 has been highlighted in a recent report by Tesco *et al.* for its potential role in AD pathology. GGA3 protein levels were found to be significantly decreased in AD brains and inversely correlated with increased levels of BACE1 (Tesco et al., 2007).

Given our earlier results confirming that endogenous BACE1 is predominantly degraded through the lysosomal pathway in hippocampal neurons, we set out out to investigate the fate of GGA3 in hippocampal neurons in response to 22C11 treatment.

5.2.5.1   22C11 treatment increases caspase-3 activity in hippocampal neurons

The Tesco *et al.* study referenced above demonstrated that GGA3 was a substrate for caspase-3 (Tesco et al., 2007). Additionally, it has previously been demonstrated in many instances that apoptosis-inducing levels of Aβ (Figure 5-S2) and 22C11 activates caspase-3 in a variety of cell lines and primary neurons (Harada and Sugimoto, 1999; Marin et al., 2000). With those results in mind, we wanted to determine whether sub-apoptotic levels of 22C11 could also lead to activation of caspase-3 in hippocampal neurons. To that end, we first analyzed the levels of cleaved caspase-3 in hippocampal neurons that were treated with a sub-lethal concentration of 22C11 (100 ng/ml) for 1 and 8 hours. After treatment, cells were harvested and cleaved caspase-3 levels were assessed by Western blot using an antibody which recognizes endogenous levels of the large fragment (17/19 kDa) of cleaved caspase-3 resulting from cleavage adjacent to Asp[175]. A significant increase in cleaved caspase-3 levels was apparent after 8 hours of treatment with 22C11 compared to control cells (Figure 5-8A).

It is often assumed that increased caspase-3 cleavage equates to increased caspase-3 activity. However, IAPs such as xIAP, c-IAP1/2 and Survivin can bind processed caspase-3 and block its activity (reviewed in Liston et al., 2003). To overcome this issue, we directly measured activity of caspase-3 in live cells using a commercially available fluorescent caspase-3 substrate, DEVD-NucView. The DEVD peptide is attached to a DNA-binding dye, which is unable to produce fluorescence in the absence of DNA. Upon entering the cell cytoplasm, it is cleaved by active caspase-3 to release the high-affinity DNA dye. The released dye migrates to the nucleus and brightly stains it. Consistent with the observed increased in cleaved caspase-3 levels, we found that hippocampal neurons treated with 22C11 showed a notable increase in fluorescence compared to control neurons (Figure 5-8B), indicative of increased DEVD cleavage by

active caspase-3. Fluorescence was significantly attenuated by pre-treating the neurons for 1 hour with the competing non-fluorescent caspase-3 inhibitor Z-DEVD-FMK (25 µM; Figure 5-8B).

Figure 5-8. 22C11 treatment results in increased cleavage and activity of caspase-3 in hippocampal neurons

Primary hippocampal neurons were treated with with 22C11 (100 ng/ml) for various time points (1 and 8 hours) before lysates were analyzed by (**A**) Western blot using an cleaved-caspase-3-specific antibody. Treatment with 22C11 resulted in increased cleavage of caspase-3 compared to vehicle (PBS)-treated neurons. (**B**) NucView assay for *in vivo* caspase-3 activity: primary hippocampal neurons cultured in chamber slides were treated with 22C11 (100 ng/ml) for 8 hours before incubating with the DEVD-NucView caspase-substrate. Upon cleavage of this substrate by caspase-3, the NucView moiety enters the nucleus and fluoresces upon binding DNA. Treated neurons showed a marked increase in fluorescence compared to vehicle (PBS)-treated neurons indicative of increased caspase-3 activity. Pre-treatment for 1 hour with the caspase inhibitor z-DEVD-FMK blocked increased NucView fluorescence, confirming that fluorescence was a result of caspase activity.

5.2.5.2   22C11 treatment increases caspase-3 dependent cleavage of GGA3

Our data above indicated increased caspase-3 activity in 22C11-treated hippocampal neurons. Since GGA3 was shown to be a caspase-3 substrate (Tesco et al., 2007), we decided to look at the state of endogenous GGA3 protein in 22C11-treated neurons. Cleavage of full-length GGA3 by active caspase-3 was shown to occur at three major

sites within its hinge domain, generating three C-terminal fragments of ~50 kDa, ~48 kDa and ~37 kDa, as well as an N-terminal fragment, which also function as a dominant negative form of GGA3 (Figure 6-9A; Tesco et al., 2007). As in our earlier experiments, hippocampal neurons were treated with a sub-apoptotic concentration of 22C11 for 8 hours. Cells were harvested and cleavage of endogenous GGA3 in hippocampal neurons, was assessed by Western blot using a commercially available antibody against the C-terminus of GGA3, which recognizes the full-length protein as well as the three C-terminal fragments (Figure 5-9B). Cells that were treated with 22C11 had increased levels of cleaved GGA3 (~37 kDa fragment) compared to control neurons (Figure 5-8C). However, we could not detect the larger ~50 kDa fragment of GGA3. One possible explanation is that the levels were below the limit of detection. This is consistent with previous reports, where the authors were also unsuccessful at detecting endogenous levels of the larger fragment in lysates from the human glioblastoma cell line, H4. Interestingly, no significant change in the levels of the ~48 kDa fragment was observed in 22C11-treated cells, suggesting a possible preference for the third cleavage site to produce the smaller fragment.

Our next objective was to confirm the role of caspase-3 in the cleavage of GGA3 in neurons. The demonstrated lack of specificity of many of the available chemical caspase inhibitors (McStay et al., 2008), persuaded us to use an siRNA to deplete the levels of endogenous caspase-3 (siCasp3). The siRNA was delivered to the neurons by linking it to the cell-penetrating peptide Penetratin-1™, which has previously been used as an effective method for delivering siRNA sequences to primary neurons (Davidson et al., 2004). We achieved about 40% knockdown of endogenous caspase-3 protein in hippocampal neurons after 8 hours Figure 5-S1). Nevertheless, this was sufficient to significantly abrogate cleavage of GGA3, as demonstrated by the reduced levels of the ~37 kDa fragment in 22C11 neurons (Figure 5-9B, C). Interestingly, basal levels of

cleaved GGA3 were not affected by treatment with siRNA alone suggesting that perhaps that other factors may play a role in regulating basal levels of GGA3.
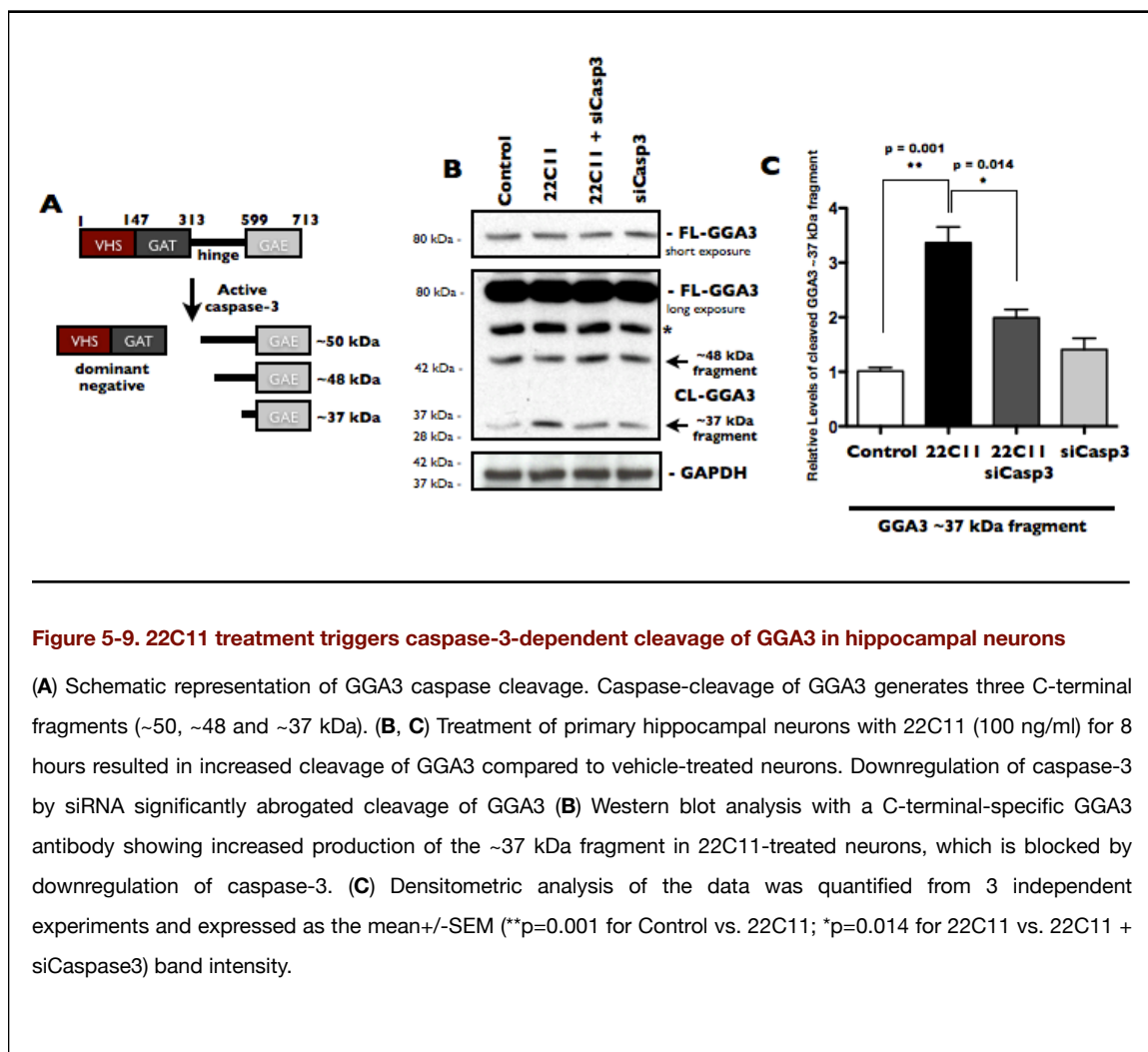
Figure 5-9. 22C11 treatment triggers caspase-3-dependent cleavage of GGA3 in hippocampal neurons

(**A**) Schematic representation of GGA3 caspase cleavage. Caspase-cleavage of GGA3 generates three C-terminal fragments (~50, ~48 and ~37 kDa). (**B**, **C**) Treatment of primary hippocampal neurons with 22C11 (100 ng/ml) for 8 hours resulted in increased cleavage of GGA3 compared to vehicle-treated neurons. Downregulation of caspase-3 by siRNA significantly abrogated cleavage of GGA3 (**B**) Western blot analysis with a C-terminal-specific GGA3 antibody showing increased production of the ~37 kDa fragment in 22C11-treated neurons, which is blocked by downregulation of caspase-3. (**C**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (**p=0.001 for Control vs. 22C11; *p=0.014 for 22C11 vs. 22C11 + siCaspase3) band intensity.

5.2.5.3   22C11-induced BACE1 and Aβ increase are caspase-3 dependent

The above results suggest a mechanism whereby 22C11 increases caspase-3 activity in hippocampal neurons, followed by increased cleavage of GGA3. Our hypothesis predicts then that depletion of caspase-3 by siRNA as above should reverse the effects of 22C11 on both BACE1 and Aβ levels. To test this prediction, we treated hippocampal neurons with a sub-apoptotic concentration of 22C11, in the presence and absence of

the caspase-3 siRNA. As in our earlier results, 22C11 treatment led to a significant increase in BACE1 and Aβ levels in hippocampal neurons (Figure 5-10A, B). The effects of 22C11 were markedly blocked in neurons with partially depleted caspase-3 levels, as indicated by a reduction in the levels of BACE1 and Aβ to near control (Figure 5-10A, B).

Figure 5-10. 22C11-induced BACE1 and Aβ increase in hippocampal neurons is caspase-3-dependent (**A**, **B**) Primary hippocampal neurons were treated with 22C11 (100 ng/ml) only or with 22C11 + siCaspase-3 for 8 hours. (**A**) Western blot and immunoprecipitation analysis showing increased levels of BACE1 and Aβ production in 22C11-treated neurons compared to vehicle (PBS)-treated neurons. Downregulation of caspase-3 blocked both BACE1 increase and Aβ production. (**B**) Densitometric analysis of BACE1 was quantified from 3 independent experiments and expressed as the mean+/-SEM (*p=0.013 for Control vs. 22C11; *p=0.052 for 22C11 vs. 22C11 + siCaspase3) band intensity. showing increased Aβ production in 22C11-treated neurons, which is blocked by downregulation of caspase-3.

Taken collectively, these results lend support for a mechanism in which 22C11 promotes BACE1 accumulation in hippocampal neurons by interfering with its normal degradation process. Activation of caspase-3 by 22C11 leads to increased cleavage of GGA3, generating its N-terminal dominant negative fragment. This process is dependent on caspase-3 as its depletion by siRNA reverses the effects of 22C11 both on BACE1 levels and Aβ production.

5.2.6 22C11 treatment leads to accumulation of BACE1 in endosomes

As mentioned above, depletion of GGA3 by siRNA impairs its normal function of targeting BACE1 to lysosomes for degradation. This leads to the accumulation of BACE1 in early endosomes (He et al., 2005; Tesco et al., 2007; Wahle et al., 2005). Since 22C11 treatment promotes GGA3 cleavage, our model would predict a similar effect. Namely, 22C11 treatment should lead to an increase of BACE1 in the endosomal compartments of treated hippocampal neurons. To test this prediction, hippocampal neurons were treated as described above with 22C11 for 8 hours. After treatment, the neurons were fixed with paraformaldehyde and stained with a monoclonal antibody against the early endosome marker EEA1 (Figure 5-11B, F, J; Mu et al., 1995) as well as a polyclonal antibody against endogenous BACE1 (Figure 5-11A, E, I). Confocal analysis of the obtained images (Figure 5-11C, G, K) revealed a marked increase in the colocalization of BACE1 and EEA1 in 22C11-treated compared to control neurons (Figure 5-11D, H). This would indicate that BACE1 is accumulating in early endosomal compartments of 22C11-treated neurons, in support of our proposed model.

We demonstrated above that the effects of 22C11 on the levels of GGA3 were dependent on caspase-3 function. Therefore, similarly, we predicted that downregulation of caspase-3 would also reverse the observed the 22C11-induced accumulation of BACE1 in endosomes. This prediction was tested in hippocampal neurons treated with 22C11 and the caspase-3 siRNA. Consistent with our model, downregulation of caspase-3 resulted in a notable reduction in the colocalization of BACE1 with EEA1 (Figure 5-11H, L), suggesting that BACE1 is prevented from accumulating in early endosomes.

Taken together, these results provide additional evidence in support of our model. 22C11 treatment blocks lysosomal degradation of BACE1, allowing it to accumulate in early endosomes. Depletion of caspase-3 in treated cells prevents this accumulation by presumably restoring normal trafficking of BACE1 to lysosomes. We wanted to test the latter prediction by attempting to detect a shift in the accumulation of BACE1 from endosomes to lysosomes using the the lysosomal markers LAMP1 and LAMP2 (Fukuda et al., 1988). However, unlike the reports by Koh *et al.* (Koh et al., 2005) we were unsuccessful at detecting any significant colocalization of endogenous BACE1 with either marker in both control and treated neurons (data not shown). One possible explanation could be due to low levels of non-degraded endogenous BACE1 in neuronal lysosomes, below the detection limit of our experiment.

Figure 5-11. Treatment with 22C11 induces accumulation of BACE1 in early endosomal compartments
Primary hippocampal neurons cultured in chamber slides were treated with 22C11 (100 ng/ml) for 8 hours. Following fixation and permeabilization, neurons were stained with antibodies for BACE1 (**A**, **E**, **I**) and the sub-cellular early endosome marker EEA1 (**B**, **F, J**) and imaged and analyzed by confocal microscopy (**C**, **G**, **K**). (**D**) Colocalization of BACE1 with EEA1 in control vehicle (PBS)-treated neurons was markedly increased in (**H**) neurons treated with 22C11 consistent with an endosome to lysosome trafficking defect. (**I-L**) Downregulation of caspase-3 by siRNA completely blocked (**L**) colocalization of BACE1 with EEA1.

5.2.7 Over-expression of human GGA3 blocks 22C11-induced Aβ increase

Since downregulation of GGA3 leads to increased BACE1 levels, our next objective was to determine whether over-expression of GGA3 could block the increase in BACE1 and Aβ production triggered by 22C11. Initially, we had wanted to test this in primary hippocampal neurons. However, because of the low transfection efficiency in primary cultures, we decided to use the previously described neuroblastoma cell line B103, stably expressing APP. These cells were treated with a sub-apoptotic concentration of 22C11 (100 ng/ml) for 8 hours and the levels of intracellular Aβ were determined by immunoprecipitation as described earlier. Similar to hippocampal neurons, B103 cells treated with 22C11 show a significant increase in both BACE1 levels and Aβ production (Figure 5-12A, B). To test GGA3 function, B103 cells were transfected with a plasmid expressing full-length human GGA3. After 48 hours, these cells were treated with the same concentration of 22C11 as above. Consistent with our proposed model, cells over-expressing GGA3 did not exhibit an increase in BACE1 levels nor Aβ production in response to 22C11 treatment.

Figure 5-12. Over-expression of human GGA3 restores normal degradation of BACE1 and blocks Aβ increase in 22C11-treated B103 cells

B103 cells stably expressing APP (B103^APP) were transfected with 4.0 µg GGA3 plasmid or 4.0 µg emplty vector and treated with 500 ng/ml of 22C11 after 48 hours. (**A**) Representative Western blots showing increased BACE1 levels in mock-transfected cells, as well as increased Aβ production. Overexpression of human GGA3 in the cells blocked both BACE1 increase and Aβ production. (**B**) Aβ levels were determined by immunoprecipation and quantified from 3 independent experiments and expressed as the mean +/- SEM (*p=0.036) band intensity.

5.3   Discussion

Much progress has been accomplished towards deciphering some of the biological functions of APP, particularly for its role in facilitating cell-cell adhesion through homo- and hetero-dimerization with the other APP family protein, namely APLP1 and APLP2. However, the focus has remained mostly on its pathogenic role in the context of Alzheimer's disease, due to it being the source of the Aβ peptide. The identification and characterization of APP-cleaving enzymes, such as secretases (BACE1 and the γ-secretase complex) and caspases have provided great insight into the complex steps involved in the proteolytic processing of APP and the production of Aβ. Whether the Aβ peptide itself is the cause of AD remains a subject for debate. The identification of several mutations in the APP and presenilin (PS) genes in early-onset familial AD seem to support this view, as these mutations have been shown promote the production of Aβ. In late-onset AD however, the picture becomes a little more nebulous. While many risk factor genes have been identified, age remains the greatest risk factor for AD. This raises several important questions: does the cleavage process of APP change with aging or in AD, and if so what triggers these changes? Do elevated BACE1 levels in the brain play a role in AD pathogenesis, and if so what causes BACE1 to become elevated? Could dimerization of APP provide some of these answers?

The exact consequences of homo-dimerization of APP on the processing of APP is not fully understood and remains a contentious topic. Introduction of a cysteine mutation in the juxtamembrane (JM) region of APP has been reported to enhance Aβ production through the formation of stable disulfide-linked APP dimers (Scheuermann et al., 2001), consistent with the observation that stable Aβ dimers can be found intracellularly *in vitro* and *in vivo* in brains (Walsh et al., 2000). However, other laboratories have reported

the opposite effect, where enhanced dimerization of APP led to decreased APP processing and decreased Aβ levels (Eggert et al., 2009; Struhl and Adachi, 2000). Reconciling this dichotomy remains difficult, but could simply be explained by differences in the manner through which APP dimerization is promoted in each model systems.

Here we show that enhanced oligomerization of endogenous APP through the use of a divalent antibody triggers the amyloidogenic pathway in cultured hippocampal neurons, resulting in a rise in the levels of intracellular Aβ. This increase in Aβ was observed under non-apoptotic conditions, at least within 48 hours of treatment. Synaptic dysfunction, as indicated by a significant reduction in synaptic markers and spine density, is observed however at these concentrations. This is an important consideration since neuronal and non-neuronal cells undergoing apoptosis have been shown to overproduce and secrete Aβ, whether triggered by staurosporine or by trophic factor withdrawal (Barnes et al., 1998; Galli et al., 1998; Gervais et al., 1999; Guo et al., 2001; LeBlanc, 1995; Matrone et al., 2008a; Sodhi et al., 2004; Tesco et al., 2003). This wide array of conditions and cell types raises questions as to whether the observed Aβ overproduction is a specific process or simply a general response to apoptotic stimuli. Our result supports the former view, but it is possible that both APP signaling and apoptosis stimuli share common pathways.

Whether driven by apoptosis or by APP signaling, enhanced Aβ production seems to correlate with high levels of BACE1. We show that the observed Aβ overproduction in treated neurons is due primarily to increased processing of APP by BACE1, but not by γ-secretase. This was reflected by a significant increase in the production of sAPPβ fragments correlating with elevated BACE1 protein levels. BACE1 levels rise in response

to physiological stress or injury, such as oxidative stress (Tamagno et al., 2002), traumatic brain injury (Blasko et al., 2004), ischemia (Wen et al., 2004), hypoxia (Zhang et al., 2007) and energy impairment (Velliquette et al., 2005). BACE1 is also increased in brains from LOAD and EOFAD patients compared to cognitively normal individuals (Fukumoto et al., 2002; Holsinger et al., 2002; Li et al., 2004; Tyler et al., 2002; Yang et al., 2003). Our results imply that in addition to age-related stress, aberrant signaling triggered by APP oligomerization may enhance levels of BACE1 and Aβ in the brain, and drive AD pathogenesis.

The exact mechanism of this up-regulation is not fully understood and hypotheses vary from transcriptional, post-transcriptional, translational and post-translational modifications of BACE1 (Faghihi et al., 2008; Hébert et al., 2008; Holsinger et al., 2002; Wen et al., 2008; Zhao et al., 2007). Our results indicate that BACE1 increase may be due, at least in part, to enhanced protein stabilization and accumulation. This is reflected by the fact that no significant changes were observed in BACE1 mRNA in treated hippocampal neurons. Alternatively, BACE1 half-life was significantly prolonged in treated neurons, persisting to near control levels after 18 hours of cycloheximide treatment. These results suggest that oligomerization of APP may trigger a signaling cascade that directly interferes with the normal degradation of BACE1 protein, thus allows BACE1 to accumulate in treated neurons. Our data shows that activation of this pathway may lead to the loss of function of the GGA3 protein. GGA family proteins are known to be involved in the trafficking of proteins, such as BACE1, which contain the DXXLL signal between different compartments, e.g. Golgi complex, endosomes and lysosomes (reviewed in Bonifacino, 2004). We demonstrated that activation of caspase-3 in treated neurons promotes cleavage of GGA3, likely generating increased amounts of the dominant negative N-terminal fragment (Tesco et al., 2007), although this was not directly established in our study. The accumulation of BACE1 in early

endosomal compartments in treated neurons was consistent with a loss of function of the GGA3 protein. Furthermore, downregulation of caspase-3 by siRNA prevented the accumulation of BACE1 in endosomes as well as an increase in intracellular Aβ. A similar effect was observed in cells over-expressing human GGA3, which did not exhibit an accumulation of BACE1 nor overproduction of Aβ.

The mechanism by which GGA3 targets some cargo to lysosomes has been shown to be ubiquitin-dependent (Puertollano and Bonifacino, 2004). While there is some evidence that BACE1 is ubiquitinated (Qing et al., 2004), future studies will be required to determine whether GGA3-dependent degradation of BACE1 requires ubiquitination or whether is occurs *via* an alternate mechanism --e.g. binding the VHS domain of GGA3. Additionally, RNAi silencing of GGA1 and GGA2 has also been shown to lead to the accumulation of BACE1 in endosomes. However, unlike GGA3, which shuttles BACE1 from endosomes to lysosomes, GGA1 and GGA2 appear to regulate retrograde transport of BACE1 from endosomes to the TGN (Wahle et al., 2005). Further studies will be required to determine whether loss of function or depletion of GGA1 and GGA2 contribute to BACE1 accumulation in our model. Finally, phosphorylation of BACE1 at Serine[498] facilitates its binding to GGA proteins (He et al., 2002; He et al., 2003; Shiba et al., 2004; von Arnim et al., 2004). However, we did not observe any changes in the phosphorylation status of BACE1 in treated neurons (data not shown).

In summary, taken collectively our results argue for a well-defined mechanism through which aberrant APP signaling can trigger amyloidogenic processing of APP, without affecting neuronal survival, as depicted in Figure 5-13.
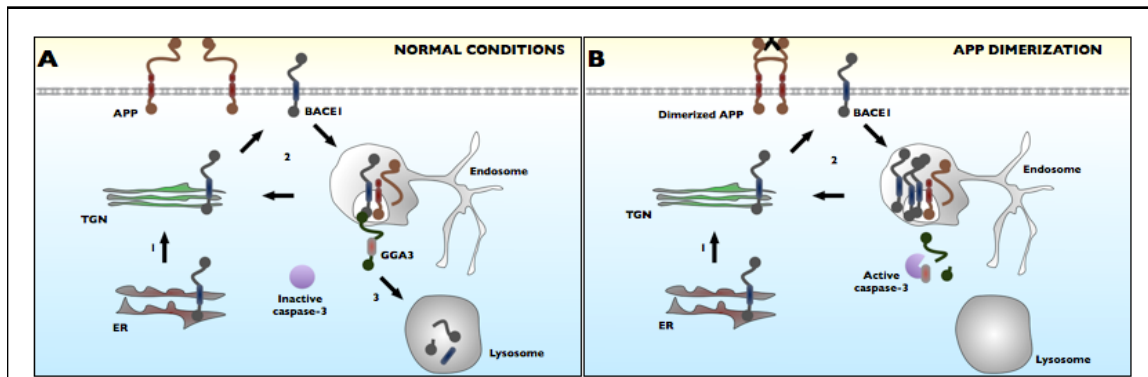
Figure 5-13 Schematic illustration of 22C11-induced BACE1 accumulation and Aβ production in neurons

Following synthesis in the endoplasmic reticulum, pro-BACE1 traffics through the trans-Golgi network (TGN) where it matures before localizing to the plasma membrane. (**A**) Under normal conditions, surface BACE1 is re-internalized through the endosomal pathway and may be recycled from endosomes to the TGN back to the plasma membrane. Alternatively, BACE1 can interact with GGA3, which targets BACE1 to lysosomes for degradation. (**B**) Dimerization of APP and activation of caspase-3 results in the cleavage of GGA3, preventing it from shuttling BACE1 to lysosomes. This allows BACE1 to accumulate in endosomes, resulting in increased cleavage of APP and Aβ production.

As we discussed earlier, alterations in synaptic density occur early in AD and strongly correlate with the cognitive decline observed in the disease (reviewed by Scheff and Price, 2006). Our results suggest that aberrant signaling through APP oligomerization is sufficient to drive synaptic dysfunction as well as promote Aβ production in hippocampal neurons. Whether, these effects are dependent on each other remains unclear. However, it raises the interesting possibility that abnormal oligomerization of APP initiates a positive feedback loop in an affected neuronal population, resulting in local synaptic dysfunction and local increases in Aβ production.

Supplemental Figure 5-S1. siRNA-mediated down-regulation of caspase-3 in hippocampal neurons after 8 hours

Primary hippocampal neurons were treated with 80 nM Penetratin-1-linked Caspase-3 siRNA (siCasp3) for 8 hours. (**A**) Representative Western blot showing a decrease in caspase-3 protein levels. (B) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (**p=0.01) band intensity.

Supplemental Figure 5-S2. Aβ treatment increases cleavage of caspase-3 in hippocampal neurons

Primary hippocampal neurons were treated with various stimuli, including oligimeric Aβ (1.0 µM), Penetratin-1linked C31 (C31-P; 100 nM), and Camptothecin (Cto; 1.0 µM) for 8 hours. Western blot analysis with a cleaved-caspase-specific antibody showing increased caspase cleavage in Aβ and Cto-treated neurons. C31 did not induce cleavage of caspase-3.

Supplemental Figure 5-S3. Expression of C99-myc fragments in HEK293 cells

HEK293 cells were transfected with increasing concentrations of pcDNA3.1-C199-myc (2.0, 4.0, 8.0, 10 and 12 µg) with lipofectamine 2000 resulting in robust expression of C99-myc fragments after 24 hours as determined by Western blot analysis using a myc-specific antibody.

CHAPTER 6: MECHANISTIC STUDIES INTO HOW APP DIMERIZATION LEADS TO SYNAPTIC DYSFUNCTION

6.1 Is the synaptotoxicity of 22C11 tied to the production of Aβ

The data presented above indicates that increasing dimerization of cell-surface APP in primary hippocampal neurons has two important consequences. First, it triggers the amyloidogenic route with consequent intracellular accumulation of Aβ, and its partial release into the culture medium (Section 5.2). Second, it triggers a significant degree of synaptic dysfunction in hippocampal neurons, as indicated by a significant loss in dendritic spine density, as well as a marked decrease in the levels of the post-synaptic markers, PSD-95 and Drebrin A (Section 4.2). Strikingly, both of these outcomes were observed in the absence of any significant cell death, which has been shown in many instances to trigger the amyloidogenic pathway (Galli et al., 1998; LeBlanc, 1995). Because Aβ is neurotoxic and contributes to apoptosis in a variety of cultured cells, it has been proposed that increased Aβ secretion causes increased cell death in susceptible neurons. This initiates a cycle in which dying neurons in turn release more Aβ, which causes additional cell death.

However, at low concentrations (nM), Aβ does not cause any significant cell death in neurons, but does significantly affect normal synaptic functions, including long-term potential (LTP) and dendritic spine maintenance. The extensive similarities between the synaptotoxic of synthetic $A\beta_{42}$ oligomers and the effects of 22C11 on hippocampal neurons led us to wonder whether these two outcomes were interdependent, specifically whether the loss of synapses triggered by 22C11 required the production of Aβ.

Previous studies have demonstrated that interruption of NGF and BDNF signaling in PC12 cells and in hippocampal neurons result in the activation of the amyloidogenic pathway and subsequence apoptotic death (Matrone et al., 2008a; Matrone et al., 2008b). In hippocampal neurons, cell death was found to be dependent on Aβ production, as it was blocked by β- and γ-secretase inhibitors as well as by an antibody directed at against the Aβ peptide (Matrone et al., 2008a).

6.1.1   *The synaptotoxic effects of 22C11 in hippocampal neurons are blocked by the anti-Aβ antibody 4G8*

Our first approach was to investigate whether an antibody-directed approach could protect the neurons against the effects of 22C11. Hippocampal neurons were pre-incubated for 1 hour with a monoclonal antibody directed at amino acids (aa) 17-24 of Aβ (clone 4G8, 2.0 µg/ml; Figure 6-1A) prior to treating with 22C11 (100 ng/ml) for 24 hours. The addition of the Aβ antibody 4G8 completely reversed the synaptotoxic effects of 22C11, as these neurons did not incur any significant loss of dendritic spines (Figure 6-1B). Protection against 22C11 was also achieved by pre-treatment with an alternative Aβ antibody directed at aa 1-16 of Aβ, but not with a C-terminal antibody directed at the C-terminal region of full-length APP (data not shown), consistent with the idea that binding Aβ blocks the synaptotoxic effects of 22C11.

Figure 6-1. 22C11-induced spine loss in hippocampal neurons is blocked by the Aβ antibody 4G8

(**A**) Schematic illustration of the relative binding site for several APP-targeting antibodies [6E10: amino acids (aa) 1-16 of Aβ; 4G8: aa 17-24 of Aβ; APP-CT: aa 643-695 of APP]. (**B**) Primary hippocampal neurons were treated for 24 hours with 22C11 (100 ng/ml) alone, or after pre-treating for 1 hour with the Aβ-specific antibody 4G8. The synaptotoxic effects of 22C11 on dendritic spines were completely blocked by the addition of 4G8. Treatment with 4G8 alone did not significantly affect dendritic spines. Dendritic spines were quantified from an average of 12 neurons from at least 3 independent experiments, and expressed as the mean+/-SEM (*p=0.038 for Control vs. 22C11; *p=0.036 for 22C11 vs. 22C11 + 4G8) number of spines/μM. (**C**) Representative image of dendrites and spines showing the neuroprotective effects of 4G8 against 22C11.

6.1.2 The synaptotoxic effects of 22C11 in hippocampal neurons are independent of caspase-3

Our earlier data suggests that increased Aβ production in neurons treated with 22C11 results from the accumulation of BACE1 in endosomes, through a mechanism that is caspase-3 dependent (Figure 5-13). Since siRNA-mediated downregulation of caspase-3 in neurons results in attenuated Aβ production, we argued that downregulation of caspase-3 should also block the synaptotoxic effects of 22C11. To test this idea, primary hippocampal neurons, were pre-treated with a Penetratin-1-linked siRNA against caspase-3 (siCasp3) for 1 hour prior to treating with 22C11 (100

ng/ml). 24 hours after treatment, neurons were harvested and the state of the synapses was evaluated by measuring post-synaptic marker PSD-95 levels by Western blot analysis. Contrary to our hypothesis, we found that downregulation of caspase-3 did not protect neurons against 22C11 (Figure 6-2A).

6.1.3 The synaptotoxic effects of 22C11 are not blocked by inhibiting production of Aβ

Since the two results above, conflicted with each other, we decided to test whether blocking production of Aβ by inhibiting γ-secretase or β-secretase activity could protect neurons against 22C11. To that end, hippocampal neurons, were pre-incubated with several inhibitors of APP secretases, including β-secretase inhibitors IV (5.0 µM) and OM99-2 (2.5 µM) as well as γ-secretase inhibitor IX (25 µM) for 1 hour prior to treating with 22C11 (100 ng/ml) for 24 hour. Once again, paradoxically, neither inhibitor protected neurons against the synaptotoxic effects of 22C11, as determined by measuring the levels of the post-synaptic protein PSD-95 (Figure 6-2B).

On the surface, these three results appear to be incompatible with each other. If Aβ production was required for the effects of 22C11, the blocking its production should have blocked the effects of 22C11. Yet, neither downregulation of caspase-3 nor secretase inhibitors demonstrated any protection. In light of these results, how do we then account for the protection conferred by the Aβ antibodies? One possibility is that the antibodies protect neurons through a different mechanism.

Figure 6-2. 22C11-induced loss of PSD-95 in hippocampal neurons is independent of caspase-3 and Aβ production

Primary hippocampal neurons were treated over the course of 24 hours with 22C11 (100 ng/ml) alone or with 22C11 + siRNA against caspase-3 (80 nM), or with 22C11 + γ-secretase inhibitor IX (25 µM) and β-secretase inhibitors IV (10 µM) and OM99-2 (5,0 µM). (**A**) Western blots showing the lack of protection against the synaptotoxic effects of 22C11 by (**B**) siRNA-mediated downregulation of caspase-3 or by (C) the inhibitors of γ-secretase and β-secretase. (data not quantified; N=2)

6.1.4 Treatment with anti-Aβ antibodies decrease the expression of cell-surface APP in hippocampal neurons

Tampellini *et al.* recently demonstrated that Aβ antibodies protect against synaptic alterations by reducing intracellular Aβ (Tampellini et al., 2007). They propose that the antibodies get internalized after binding the extracellular portion of APP. It is likely 4G8 protects against 22C11 through a similar mechanism. To test this hypothesis, we measured the levels of APP expressed at the cell surface of primary hippocampal neurons that had been incubated with several Aβ antibodies [4G8 and 6E10 (2.0 µg); APP-CT: 2.0 µg/ml)] for 2 hours prior to analysis. Cell surface proteins were biotinylated and precipitated by incubating streptavidin coated beads. Western blot analysis of the precipitated protein with the APP antibody 22C11 (Figure 6-3A) revealed a significant reduction in the levels of surface-expressed APP in neurons incubated with the Aβ

antibodies 4G8 and 6E10 (Figure 6-3B). The monoclonal antibody directed against the C-terminal portion of APP (APP-CT) did not affect cell-surface APP, consistent with the idea that Aβ antibodies may become endocytosed together with APP upon binding it. However, this remains to be demonstrated as we do not have any evidence confirming any internalization of the Aβ antibodies.

Figure 6-3. Treatment with Aβ antibodies decreases cell-surface APP in hippocampal neurons

Primary hippocampal neurons were incubated for 1 hour with various antibodies against several epitopes on APP [6E10: amino acids (aa) 1-16 of Aβ; 4G8: aa 17-24 of Aβ; APP-CT: aa 643-695 of APP]. Cell surface proteins were biotinylated and immunoprecipitated with streptavidin-coated beads. (**A**) Western blot analysis of isolated cell-surface proteins with APP-antibody 22C11 showed a significant decrease in cell-surface APP in neurons treated with 4G8 and 6E10. No significant changes were observed in neurons treated with the APP C-terminal antibody APP-CT. (**B**) Densitometric analysis of the images was quantified from 3 independent experiments (N=2 for 6E10) and expressed as the relative mean+/-SEM (**p=0.003 for 4G8) band intensity.

6.2   Alternative mechanism of 22C11-induced synaptotoxicity

If endogenous rat Aβ is not the sole cause of the synaptotoxic effects of 22C11, what then is the intracellular mechanism? In addition to amyloidogenic and non-amyloidogenic processing, APP can be cleaved by caspases in its C-terminal region (Aspartate[664] of APP[695]) to produce an intracellular cytotoxic fragment termed C31 (Section 1.8.4; Pellegrini et al., 1999). Several caspases have been implicated in the

cleavage of APP, including caspase-3, -6, -8 and -9 (Galvan et al., 2002; Lu et al., 2000; Lu et al., 2003b; Pellegrini et al., 1999). This suggests that C31 could be generated by a host of stimuli that activate one or more of these caspases. One such insult is the Aβ peptide, which has been shown to promote C-terminal cleavage of APP and production of C31 (Lu et al., 2003b). Since dimerization of APP by Aβ is a key mediator of this cleavage event (Shaked et al., 2006), we postulated that 22C11 could also induce APP cleavage and C31 production.

6.2.1 22C11 treatment promotes caspase-dependent C-terminal cleavage of APP at Asp^{664}

To test this hypothesis, cultured hippocampal neurons were treated with 22C11 (100 ng/ml) for 24 hours. After treatment, the cells were then harvested, lysed and analyzed by Western blot using an antibody directed at the newly generated APP C-terminal fragment (APP-neo; Figure 6-4A). Neurons that were treated with 22C11 showed increased cleavage of APP as indicated by the higher levels of APP-neo in treated neurons compared to those treated with vehicle (PBS) (Figure 6-4B). This increase in APP-neo was comparable to that seen in neurons that were treated with oligomeric Aβ (300 nM; Figure 6-4B). 22C11-induced cleavage of APP was abrogated by the addition of pan-caspase inhibitor z-VAD-FMK (Figure 6-4B) confirming that APP is cleaved by caspases to produce the C31 fragment.

Figure 6-4. 22C11 treatment triggers caspase-dependent cleavage of APP at Asp^{664} in hippocampal neurons
(**A**) Schematic illustration of caspase-mediated cleavage of APP and generation of C31 fragments as well as the newly generated APP-neo epitope. (**B**) Hippocampal neurons were treated with oligomeric Aβ (1.0 nM) and with 22C11 (100 ng/ml) alone or with 22C11 + caspase-inhibitor z-VAD-FMK (zVAD; 25 µM) for 24 hours. Western blot analysis with an antibody directed against the APP-neo epitope revealed an increase in cleavage of APP. Co-treatment with the general caspase inhibitor zVAD blocked cleavage of APP.

This result indicates that in addition to stimulating Aβ production in hippocampal neurons, 22C11 also drives caspase-mediated cleavage of APP and presumably generation of the C31 fragment. Unfortunately, our efforts to isolate or directly detect the C31 fragment *in vitro* or *in vivo* have yielded mixed results at best. One possible explanation is that the newly generated C31 fragment is rapidly degraded before it can be observed. This would suggest that cleaved APP is somehow inducing the observed toxicity (Nishimura et al., 2002) and not C31 directly. However, the evidence for this alternative mechanism remains scarce.
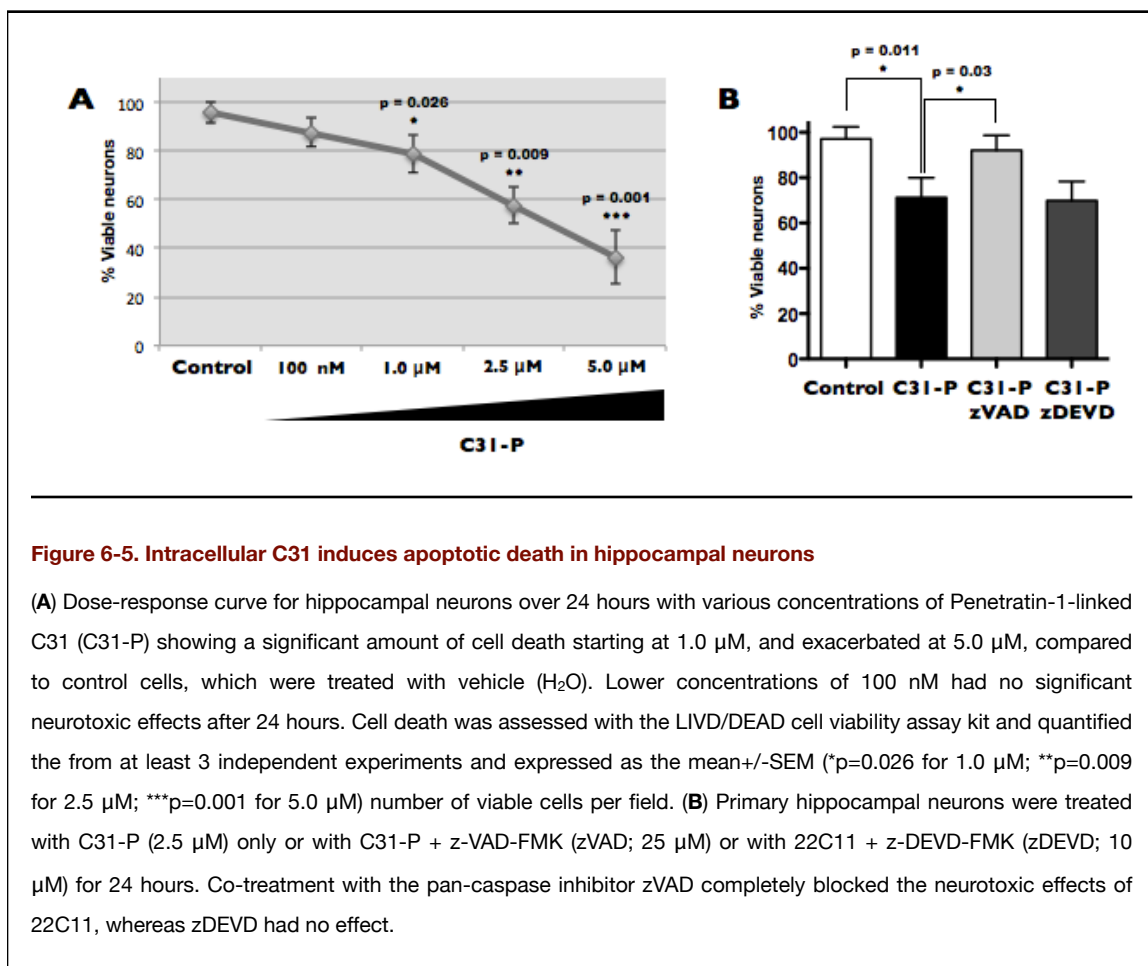
6.2.2   C31 mimics the effects of Aβ and 22C11 on hippocampal neurons

6.2.2.1   Intracellular C31 induces apoptotic death in a dose-dependent manner in hippocampal neurons

An alternative explanation is that C31 undergoes a rapid post-cleavage modification, such as phosphorylation (Ando et al., 1999), or further cleavage (Bertrand et al., 2001),

which precludes us from detecting it using the C-terminal APP antibodies at our disposal. This is supported by the fact intracellular C31 in itself is sufficient to trigger apoptotic death in a wide variety of neuronal and non-neuronal cells (Galvan et al., 2002; Kim et al., 2003; Lu et al., 2000; Lu et al., 2003a; Lu et al., 2003b). When we tested the neurotoxicity of C31 on hippocampal neurons, our results were consistent with the referenced reports.

To deliver the C31 peptide to the neurons, a synthetic C31 fragment containing an extra C-terminal cysteine residue was linked to the cell-penetrating peptide Penetratin-1™ (Pen1; Galvan et al., 2002). After 24 hours, Pen1-linked C31 (C31-P) revealed to be toxic to hippocampal neurons in a dose-dependent manner (Figure 6-5A). Neither unlinked C31 nor Pen1 had any observable affect on the viability of the neurons, even after 48 hours of exposure (data not shown). Additionally, C31-P-induced cell death was blocked by the pan-caspase inhibitor z-VAD-FMK (25 µM), but interestingly not by z-DEVD-FMK (10 µM) suggesting that one or more caspases other than caspase-3 may be required for C31-induced death (Figure 6-5B). In fact, when we looked at caspase-3 in hippocampal neurons in response to C31-P (100 nM), we did not detect any increased cleavage of caspase-3 after 8 hours, as determined by Western blot analysis (Figure 6-S1A), nor any increased DEVD cleavage indicated by the NucView assay. Robust caspase-3 cleavage and activity was observed at that time point with both oligomeric Aβ (1.0 µM) and camptothecin (1.0 µM).

Figure 6-5. Intracellular C31 induces apoptotic death in hippocampal neurons

(**A**) Dose-response curve for hippocampal neurons over 24 hours with various concentrations of Penetratin-1-linked C31 (C31-P) showing a significant amount of cell death starting at 1.0 µM, and exacerbated at 5.0 µM, compared to control cells, which were treated with vehicle (H_2O). Lower concentrations of 100 nM had no significant neurotoxic effects after 24 hours. Cell death was assessed with the LIVD/DEAD cell viability assay kit and quantified the from at least 3 independent experiments and expressed as the mean+/-SEM (*p=0.026 for 1.0 µM; **p=0.009 for 2.5 µM; ***p=0.001 for 5.0 µM) number of viable cells per field. (**B**) Primary hippocampal neurons were treated with C31-P (2.5 µM) only or with C31-P + z-VAD-FMK (zVAD; 25 µM) or with 22C11 + z-DEVD-FMK (zDEVD; 10 µM) for 24 hours. Co-treatment with the pan-caspase inhibitor zVAD completely blocked the neurotoxic effects of 22C11, whereas zDEVD had no effect.

6.2.2.2 Sub-apoptotic concentration of intracellular C31 induces synaptic dysfunction in hippocampal neurons

Having established a dose-response curve for C31 in hippocampal neurons, we proceeded to investigate whether a sub-apoptotic concentration of C31-P could induce synaptic alterations in hippocampal neurons. To test this hypothesis, primary hippocampal neurons were treated with C31-P (100 nM) for 24 hours, after which they were fixed and stained with diOlistic dye as described earlier for dendritic spine analysis. Similar to Aβ and 22C11, we found a significant decrease in dendritic spine density in C31-P-treated neurons compared to vehicle (H_2O)-treated ones (Figure 6-6A,

B). Western blot analysis of lysates from treated neurons revealed a concomitant decrease in the levels of both dendritic spine markers PSD-95 and Drebrin A (Figure 6-6C-E).

Figure 6-6. Intracellular C31 induces loss of dendritic spines in hippocampal neurons

Treatment of hippocampal neurons for 24 hours with Penetratin-1-linked C31 (C31-P, 100 nM) resulted in a significant decrease in dendritic spine density, compared to vehicle (H_2O)-treated control neurons. Spine loss were measured by direct count of dendritic spines (**A**, **B**) and by measuring the levels of two dendritic spine markers, PSD-95 (**C**, **E**) and Drebrin A (**C**, **D**). (**A**) Dendritic spines were quantified from an average of 12 neurons taken from at least 3 independent experiments, and expressed as the mean+/-SEM (***$p<0.001$) number of spines/μM. (**B**) Representative image showing a decrease in dendritic spines in C31-P-treated neurons compared to control neurons. (**C**) Representative Western blots showing a decrease in the synaptic markers, PSD95 and Drebrin A in C31-P-treated neurons. (**D**, **E**) Densitometric analysis of the data was quantified from 3 independent experiments and expressed as the mean+/-SEM (*$p=0.026$ for Drebrin A; **$p=0.008$ for PSD-95) band intensity.
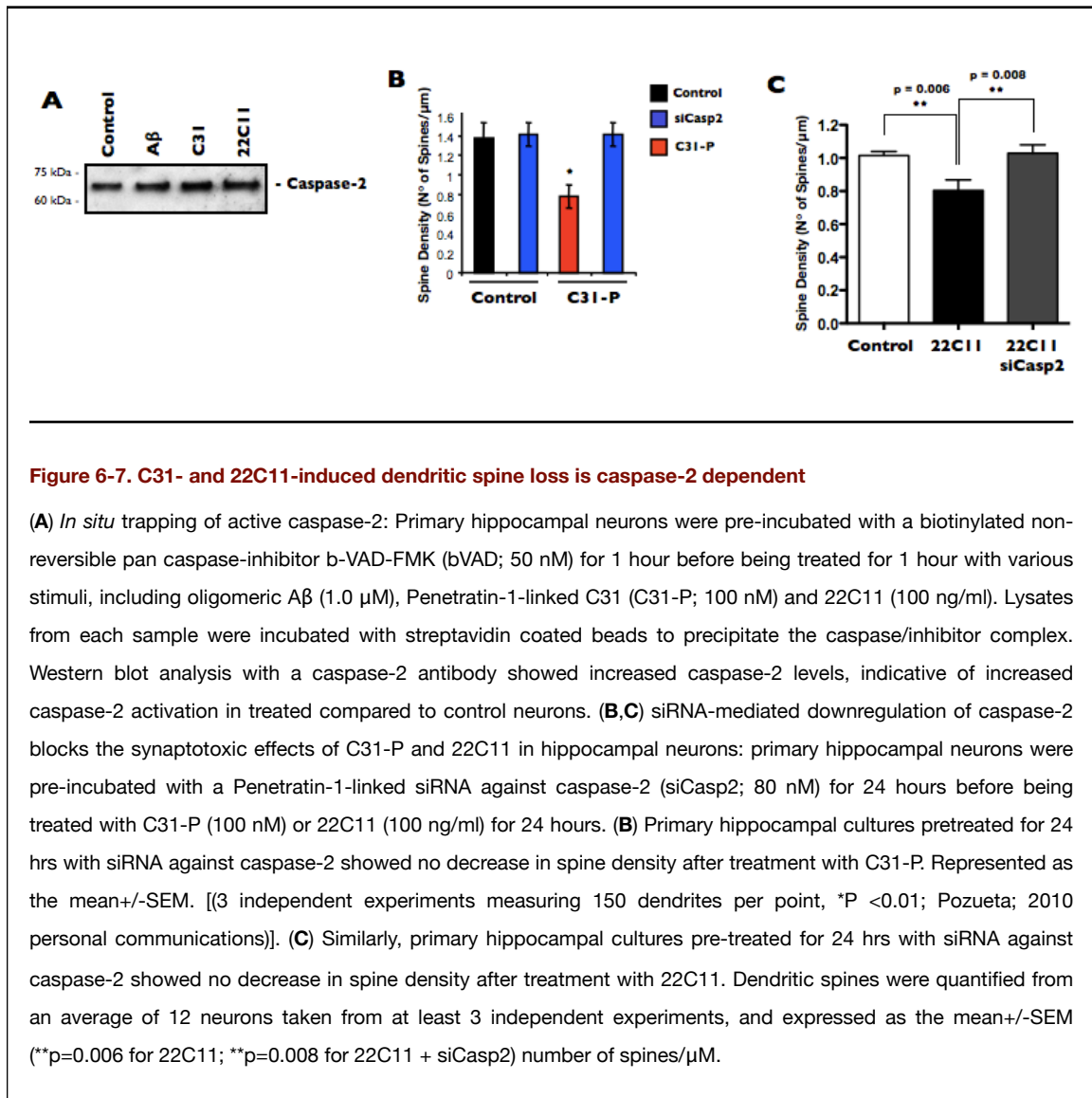
6.2.3   The synaptotoxic effects of intracellular C31 are caspase-2 dependent

Our earlier results suggested C31-P-induced death was independent of caspase-3. Interestingly, work from our lab had pointed at caspase-2 as the critical caspase responsible for mediating cell death and synaptic dysfunction in hippocampal neurons by oligomeric Aβ (Troy et al., 2000). This prompted us to also test whether the effects of C31 were also dependent on caspase-2. We first tested whether C31 could activate caspase-2 in hippocampal neurons. We used the previously described *in situ* trapping

of active caspase technique to assess caspase-2 activity (Tu et al., 2006). Primary hippocampal neurons were pre-incubated for 2 hour with a biotinylated irreversible caspase inhibitor (b-VAD-FMK; 80 nM). Neurons were then treated with the required stimuli [oligomeric Aβ (300 nM); C31-P (100 nM); 22C11 (100 ng/ml) for 1 hour before harvesting. Active caspases bind b-VAD-FMK in an irreversible manner, allowing the whole complex (bVAD/caspase) to be precipitated out using streptavidin coated beads and analyzed by Western blot with the appropriate antibody for the caspase of interest. In our case, immunoblotting with caspase-2 specific antibody revealed a strong activation of caspase-2 in hippocampal neurons treated with all three stimuli, namely oligomeric Aβ, C31-P and 22C11 (Figure 6-7A).

Next, we focused on addressing whether the effects of C31 were dependent of caspase-2. To test this hypothesis, we once again opted to downregulate caspase-2 through the use of siRNA in order to avoid possible specificity issues with chemical caspase inhibitors. Treatment of primary hippocampal neurons with Penetratin-1-linked caspase-2 siRNA (siCasp2, 80 nM; Troy et al., 2000) resulted in robust downregulation of caspase-2 after 24 hours, which persisted up to 48 hours after the start of treatment (Figure 6-S2). Neurons were treated with C31-P (100 nM) at the 24 time point after addition of Casp2 for an additional 24 hours. DiOlistic labeling of neurons allowed us to directly count dendritic spines, which showed that neurons with depleted levels of caspase-2 were completely resistant to effects of C31 (Figure 6-7B; Pozueta, 2010, personal communications) compared to control neurons, which suffered a significant amount of spine loss in response to C31-P. Downregulation of caspase-2 did not otherwise affect dendritic spines in control neurons. Similarly, we saw a significant degree of protection against 22C11 (100 ng/ml) by caspase-2 downregulation (Figure 6-7C).

Figure 6-7. C31- and 22C11-induced dendritic spine loss is caspase-2 dependent

(**A**) *In situ* trapping of active caspase-2: Primary hippocampal neurons were pre-incubated with a biotinylated non-reversible pan caspase-inhibitor b-VAD-FMK (bVAD; 50 nM) for 1 hour before being treated for 1 hour with various stimuli, including oligomeric Aβ (1.0 μM), Penetratin-1-linked C31 (C31-P; 100 nM) and 22C11 (100 ng/ml). Lysates from each sample were incubated with streptavidin coated beads to precipitate the caspase/inhibitor complex. Western blot analysis with a caspase-2 antibody showed increased caspase-2 levels, indicative of increased caspase-2 activation in treated compared to control neurons. (**B,C**) siRNA-mediated downregulation of caspase-2 blocks the synaptotoxic effects of C31-P and 22C11 in hippocampal neurons: primary hippocampal neurons were pre-incubated with a Penetratin-1-linked siRNA against caspase-2 (siCasp2; 80 nM) for 24 hours before being treated with C31-P (100 nM) or 22C11 (100 ng/ml) for 24 hours. (**B**) Primary hippocampal cultures pretreated for 24 hrs with siRNA against caspase-2 showed no decrease in spine density after treatment with C31-P. Represented as the mean+/-SEM. [(3 independent experiments measuring 150 dendrites per point, *P <0.01; Pozueta; 2010 personal communications)]. (**C**) Similarly, primary hippocampal cultures pre-treated for 24 hrs with siRNA against caspase-2 showed no decrease in spine density after treatment with 22C11. Dendritic spines were quantified from an average of 12 neurons taken from at least 3 independent experiments, and expressed as the mean+/-SEM (**p=0.006 for 22C11; **p=0.008 for 22C11 + siCasp2) number of spines/μM.

6.3   Discussion

6.3.1   Rat versus human Aβ

Whether Aβ antibodies protect by downregulating cell-surface APP or by binding up intracellular Aβ remain a difficult question to answer in system other than human neurons, specifically in rat and murine neurons One very important distinction between

human APP and murine and rat APP is in the amino acid sequence of the Aβ peptide: rodent Aβ$_{42}$ contains three amino acid variations of the human peptide sequence: Arg[5], Tyr[10] and His[13] are substituted by Gly, Phe and Arg respectively (Figure 6-S3A; Kowalik-Jankowska et al., 2002). This histidine residue in the human sequence has been shown to be critical for the aggregation of Aβ, especially in the presence of metal ions such as copper (Liu et al., 1999). In fact, it has been shown that rodent Aβ does not form fibrillar aggregates in the brain due to its lower tendency to form β-sheet structures *in vivo* (Dyrks et al., 1993; Otvos et al., 1993). Because of these differences, it is widely believed that rodent Aβ does not possess the same toxic properties as human Aβ$_{42}$. Interestingly, using synthetic rat Aβ in our standard preparation of oligomeric Aβ yields noticeably different species (Figure 6-S3B). However, the results from Matrone and colleagues suggest that rodent Aβ may be at least a contributor to apoptotic death (Matrone et al., 2008a; Matrone et al., 2008b). Nevertheless, in our system, it is possible that since apoptosis is avoided, endogenous rat Aβ does not reach sufficiently high levels to correctly aggregate to contribute to the synaptotoxic effects of 22C11.
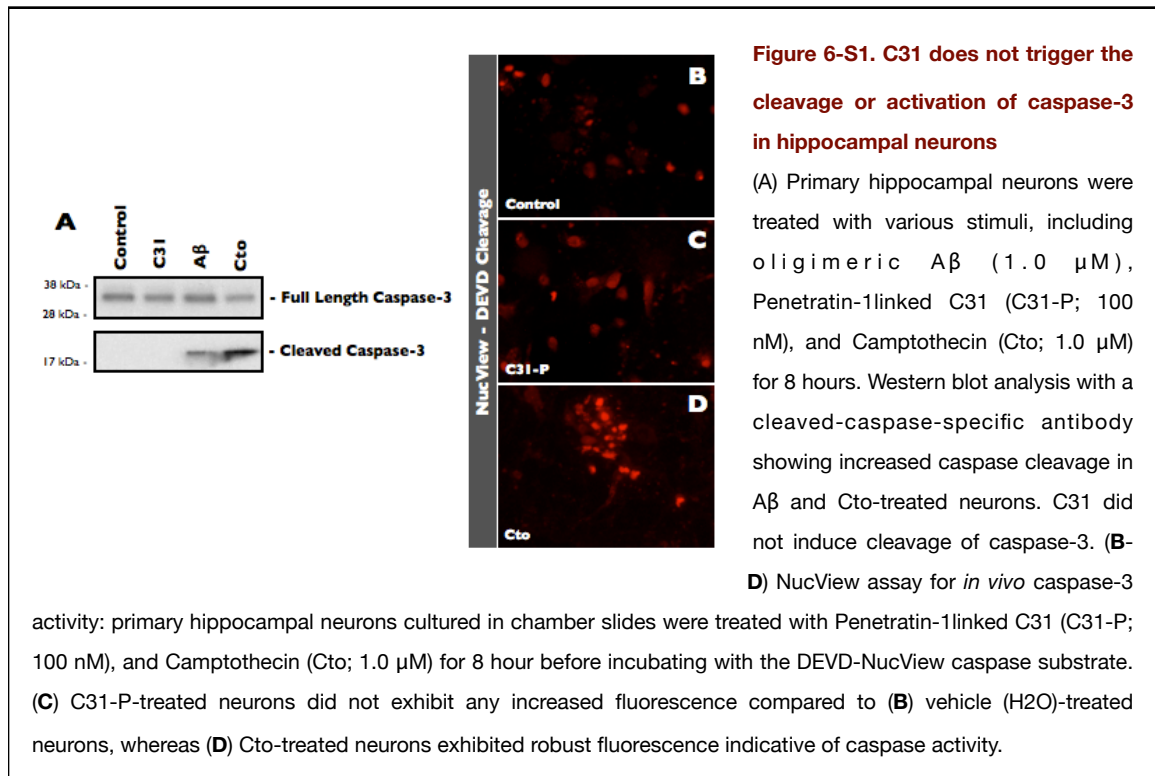
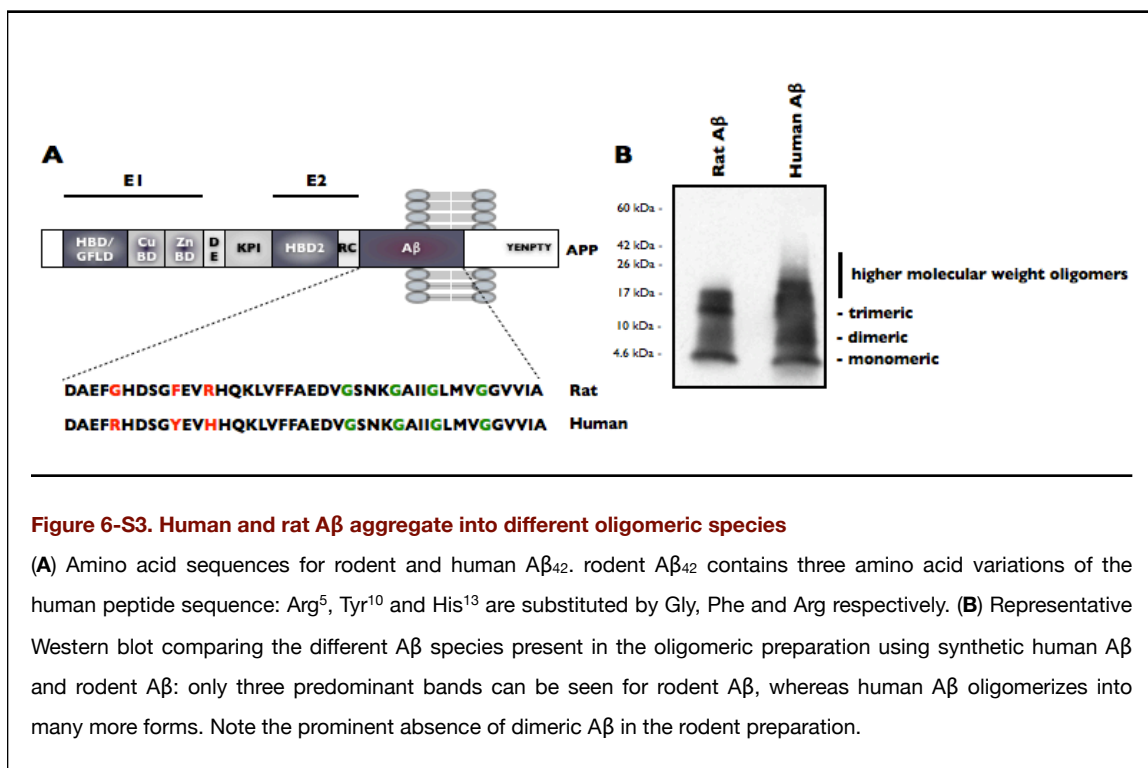### 6.3.2   C31 as intracellular messenger for 22C11/Aβ signaling

Whether induced by 22C11 or Aβ, one important aspect is to understand the signaling pathway that are activated by dimerization of APP. We have provided some evidence to suggest that caspase-mediated cleavage of APP and production of C31 may play a critical role in relaying the signal from an extracellular stimulus -- dimerization of the extracellular region of APP -- to an intracellular messenger -- C31. We showed that introduction of this fragment is sufficient to trigger a wide variety of effects, ranging from apoptotic cell death at high concentrations (1.0 μM) to synaptic dysfunction at lower concentrations (100 nM). Furthermore, we showed that caspase-2 is essential for

the effects of C31 and both 22C11 and Aβ, consistent with a model in which C31 acts downstream to those two stimuli. Unfortunately, as we alluded to earlier, the exact caspase responsible for cleaving APP remains a mystery. Caspases -8 and -9 have been posited as potential candidates, but our results suggest otherwise. Downregulation of caspase-8 and -9 where either one can cleave APP in the absence of other. Whether downregulation of both can protect against Aβ and 22C11 remains to be seen. Another potential candidate to cleave APP is caspase-6 (Pellegrini et al., 1999), but it has not been evaluated yet in our studies.

Finally, there is the question of the mechanism of C31 itself. The most commonly assigned mechanism is a transcriptional one. The YENPTY motif contained within C31 allows it to interact with various adaptor proteins, including Mint-1/X11a (and the family members Mint-2 and Mint-3), Fe65 (as well as Fe65-like proteins, Fe65L1 and Fe65L2) and c-Jun N-terminal kinase (JNK)-interacting protein (JIP), through the phosphotyrosine-binding (PTB) domain (Ando et al., 1999; Kimberly et al., 2005; Ramelot and Nicholson, 2001). Of particular interest is the interaction of APP C-terminal fragments with Fe65. APP intracellular C-terminal domain (AICD) fragment (C57/C59) has been demonstrated to induce transcriptional activation in combination with Fe65 and Tip60, a histone acetyltransferase, using a reporter gene system (Cao and Sudhof, 2001). Interestingly, C31 was also shown to be able to induce expression of genes, and one of the genes identified was Gsk3β (Kim et al., 2003). In addition to driving Gsk3β expression, they demonstrated that both AICD and C31 could not only induce cell death but could also induce phosphorylation of Tau (AT8), which are two hallmarks associated with AD. Further studies will be needed to clarify C31-mediated signaling.
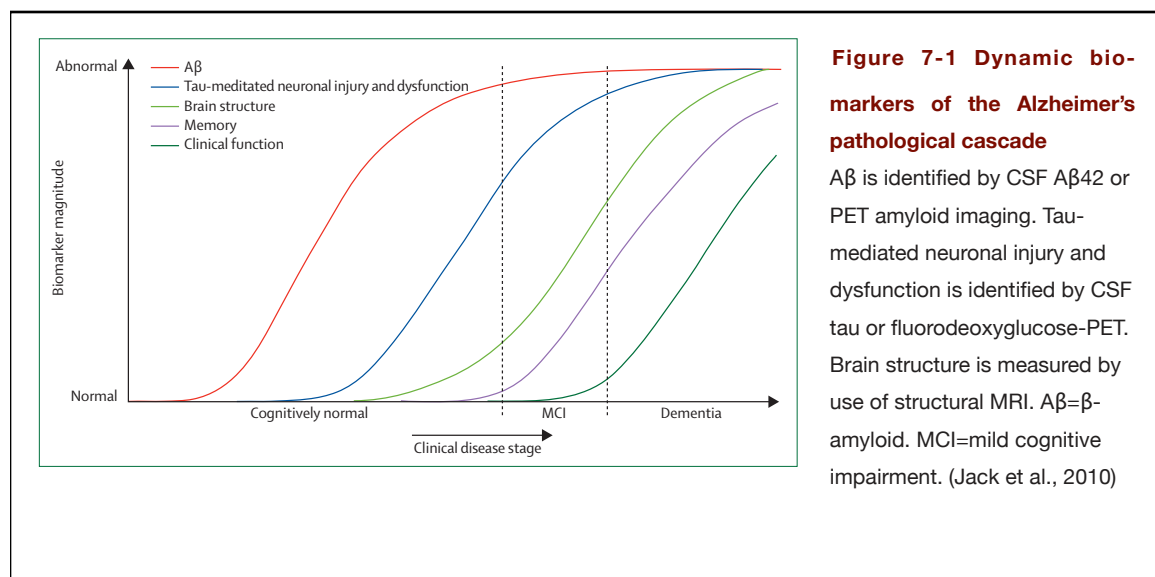
Figure 6-S1. C31 does not trigger the cleavage or activation of caspase-3 in hippocampal neurons

(A) Primary hippocampal neurons were treated with various stimuli, including oligimeric Aβ (1.0 µM), Penetratin-1linked C31 (C31-P; 100 nM), and Camptothecin (Cto; 1.0 µM) for 8 hours. Western blot analysis with a cleaved-caspase-specific antibody showing increased caspase cleavage in Aβ and Cto-treated neurons. C31 did not induce cleavage of caspase-3. (**B-D**) NucView assay for *in vivo* caspase-3 activity: primary hippocampal neurons cultured in chamber slides were treated with Penetratin-1linked C31 (C31-P; 100 nM), and Camptothecin (Cto; 1.0 µM) for 8 hour before incubating with the DEVD-NucView caspase substrate. (**C**) C31-P-treated neurons did not exhibit any increased fluorescence compared to (**B**) vehicle (H2O)-treated neurons, whereas (**D**) Cto-treated neurons exhibited robust fluorescence indicative of caspase activity.

Figure 6-S2. Time-course of siRNA-mediated caspase-2 down-regulation in hippocampal neurons

(**A**) Rat hippocampal primary cultures treated with 80 nM Penetratin linked siRNA against Casp2 show a time dependent decrease in Casp2 levels. Representative immunoblot for Casp2 levels. (**B**) Densitometric analysis of Casp2 levels shows a 60% reduction of Casp2 by 12h. During the following 24 hours Casp2 levels are maintain below 40% (Basal levels of Casp2 are indicated as 100%, n=3 independent experiments). [Pozueta et al.,(submitted)]

Figure 6-S3. Human and rat Aβ aggregate into different oligomeric species

(**A**) Amino acid sequences for rodent and human Aβ$_{42}$. rodent Aβ$_{42}$ contains three amino acid variations of the human peptide sequence: Arg^5, Tyr^{10} and His^{13} are substituted by Gly, Phe and Arg respectively. (**B**) Representative Western blot comparing the different Aβ species present in the oligomeric preparation using synthetic human Aβ and rodent Aβ: only three predominant bands can be seen for rodent Aβ, whereas human Aβ oligomerizes into many more forms. Note the prominent absence of dimeric Aβ in the rodent preparation.

CHAPTER 7: PERSPECTIVES AND FUTURE AIMS

7.1 Aβ production in Alzheimer's disease (AD)

Over the past few decades, extensive research efforts have been undertaken to understand the processing of the amyloid precursor protein (APP). Secretases, such as BACE1 and the γ-secretase complex have been identified and characterized, as well as novel caspase-cleavage site (i.e. Asp^{664}). However, while the "cutters" are known, we still do not fully understand the mechanisms that regulate these different cleavage processes, let alone how they might be dysregulated in neurodegenerative diseases, such as Alzheimer's disease (AD). Indeed, a widely accepted assumption is that AD begins with abnormal processing of APP, which leads to excess production or reduced clearance of Aβ in the brain (Hardy and Selkoe, 2002). This view is supported by the fact that all known form of autosomal-dominant AD to date involve genes that either encode for APP itself, or for the γ-secretase subunits (PS1 and PS2) directly lead to increased Aβ production. It has been speculated that it is the Aβ oligomers (Klein et al., 2004), through yet unclear mechanisms, that initiate a pathogenetic cascade characterized by abnormal tau aggregation, synaptic function failure and neuronal death (Oddo et al., 2006).

This raises an important question: if aberrant APP processing is the cause of early-onset familial AD (EOFAD), could aberrant APP processing also cause late-onset AD (LOAD)? Many factors that directly influence APP processing have been proposed to play a role in LOAD, including risk factor genes (e.g. PICALM; Harold et al., 2009), environmental factors (e.g. metal ions; Maynard et al., 2005; Walton and Wang, 2009), epigenetics (reviewed in Chouliaras et al., 2010). While there is ample evidence to

suggest that these may play an important role in AD pathogenesis, it remains that aging represents the highest risk factor for LOAD. This raises the possibility that APP processing may be altered and dysregulated over time with aging. Recent imaging studies have suggested a temporal ordering of biomarker abnormalities, reflective of the disease progression (Ingelsson et al., 2004; Jack et al., 2010). This has lead to a hypothetical model (Figure 7-1) in which biomarkers of Aβ deposition become abnormal early, before the onset of neurodegeneration and clinical symptoms, consistent with the idea of gradual altered APP processing. Biomarkers of neuronal injury, dysfunction, and neuronal degeneration become abnormal later in the disease.

Figure 7-1 Dynamic biomarkers of the Alzheimer's pathological cascade
Aβ is identified by CSF Aβ42 or PET amyloid imaging. Tau-mediated neuronal injury and dysfunction is identified by CSF tau or fluorodeoxyglucose-PET. Brain structure is measured by use of structural MRI. Aβ=β-amyloid. MCI=mild cognitive impairment. (Jack et al., 2010)

7.2. APP dimerization and Aβ production - Implications for AD

Recently, a new picture has started to emerge, which underscores the importance of APP dimerization, whether it is in a physiological context, as in promoting cell-cell-adhesion, or in a pathological context, as in influencing APP cleavage and the release of Aβ. APP homo-dimerization is driven by motifs present in the extracellular domain, as well as in the juxtamembrane (JM) and transmembrane (TM) domains of the molecule.

Strikingly, one of the structural motifs involved in the dimerization of APP is also responsible for the aggregation of Aβ peptides into proto-fibrillar structures, namely the GxxxG motif (Sato et al., 2006). Glycine residues within this motif not only allow for protein-protein interactions in the α-helical TM domain, but facilitate the formation of globular toxic forms of Aβ aggregates. Could homo-dimerization of APP play a critical role in the pathogenesis of AD? Our results along with several others (Kaden et al., 2008; Kaden et al., 2009; Munter et al., 2010; Munter et al., 2007) suggest that it may be the case.

We found that inducing APP multimerization through an antibody-mediated approach perfectly mimicked the dose-dependent neurotoxic effects of oligomeric Aβ in hippocampal neurons. At high concentrations, the antibody induced apoptotic death in neurons, whereas low sub-apoptotic concentrations triggered significant synaptic dysfunction, as reflected by a marked loss of dendritic spines. The exact mechanism through which this occurs remains unclear, however several lines of evidence (Galvan et al., 2002; Lu et al., 2000; Lu et al., 2003a; Lu et al., 2003b) including ours suggest that dimerization of APP initiates a caspase-mediated cleavage of the C-terminal end of APP to release a cytotoxic fragment termed C31. Our results also showed that this fragment recapitulates the effects oligomeric Aβ on hippocampal neurons. Moreover, in addition to adversely affecting the viability and synaptic functions of hippocampal neurons, our results suggest that multimerization of APP can also drive the processing of APP and the production of Aβ. Taken together, our data hints at the occurrence of a positive feedback loop involving Aβ and APP. According to this hypothetical model, as an individual ages, small local variations in the extent of APP multimers could not only adversely affect synaptic functions in a few neurons, but also drive the production of intraneuronal Aβ. As this Aβ is secreted, it spreads to nearby neurons thereby

perpetuating the pathogenic cycle. Over time, these small changes could continue to snowball until the advent of noticeable cognitive impairment. It is not yet known whether APP dimerization is increased in AD patients. Future fluorescent probes designed to measure APP oligomerization could provide a useful means to evaluate the aggregation state of cell-surface APP in a patient's brain and may eventually serve as a biomarker for early detection.

7.3   What factors influences APP dimerization in the brain?

In a more general perspective, one is led to wonder whether any type of molecule capable of promoting the pathogenic dimerization of APP could initiate this cascade and contribute to its amyloidogenic processing or if there are specific molecules involved in this process. Of the many binding partners for APP, one class of molecules that is of particular interest in AD are proteoglycans (PGs), more specifically heparan sulfate proteogylcans (HSPGs; reviewed in van Horssen et al., 2003). Interestingly, colocalization of carbohydrates and amyloid deposits -- hence the name amyloid -- were first described in the 19th century by Virchow (Virchow, 1853), but it was not until the 1970s that details about these molecules were studied and the relationship between PGs and amyloid deposits became a focus of interest.

HSPGs can be classified into two main families: those of the extracellular matrix, including perlecan, agrin and collagen XVIII; and those of the cell surface, such as syndecans and glypicans. Snow and his colleagues were the first to report the presence of HSPGs in diffuse and neuritic plaques by the use of immunohistochemistry (Snow et al., 1988; Snow et al., 1990). Since then, it has been reported that both intact HSPG and the core protein bind the brain specific isoform APP[695] with similar affinities (Narindrasorasak et al., 1991).

The question remains whether deposition of HSPGs in AD lesions is preceded by accumulation of Aβ or vice versa. In the brains of adolescent patients with Down's syndrome, antibodies directed against HSPGs stain diffuse primitive SP; the accumulation of proteoglycans seems to be an early event in the formation of SP (Snow et al., 1990). Additionally, accumulation of glycosaminoglycans have been shown to occur at about the same time and the same location as amyloid deposits in experimental amyloidosis (Snow and Kisilevsky, 1985). One explanation for these findings could be that the presence of Aβ affects the biosynthesis of HSPGs. Alternatively, HSPGs could promote the conversion of non-fibrillary Aβ into fibrillary Aβ, as is the case for perlecan and agrin (Castillo et al., 1997; Snow et al., 1994).

Another possible explanation could be that the interaction between APP and HSPGs might influence the generation of Aβ from APP. Dahms and colleagues recently reported that the isolated E1 domain of APP from tightly bound dimers upon interacting with another glycosaminoglycan, heparin (Dahms et al., 2010). This suggests that full-length APP may also dimerizes upon interacting with heparin (Gralle et al., 2006) or perhaps HSPGs. In support of this view, the addition of heparinase has been shown to disrupt cell-surface APP dimers in an APP over-expressing model (Gralle et al., 2009). Further studies are required to demonstrate a role for HSPGs in promoting APP dimerization. However, it is worth noting that glypican and an unidentified HSPG have also been shown to bind with high affinity the N1 domain of APP (Buee et al., 1993a; Williamson et al., 1996; Williamson et al., 1995).

It is our hope that the studies presented here may help shed some light into some of biggest unanswered questions in the field of Alzheimer's disease, namely how the disease begins and how the mechanism(s) regulating Aβ production become perturbed over time in an individual's brain and how this imbalance may lead to memory

impairments. Ultimately, a thorough understanding of APP processing may lead to novel therapeutic targets, designed to block dimerization of APP and to selectively lower Aβ levels in the brain. As a proof of concept, non-steroidal anti-inflammatory drugs (NSAID), such as sulindac sulfide and indomethacin, which are known to specifically modulate APP processing and to lower the levels of Aβ (Weggen et al., 2001), where recently shown to interfere with APP transmembrane dimerization (Richter et al., 2010). High-throughput screen to identify and develop novel APP dimer breaker may be promising avenue for therapy.

APPENDIX I: PLASMID SEQUENCES

1. pcDNA3.1-C100

2. pSG5L-Flag-HA-GGA3

3. pcDNA3.1-APP-dendra2
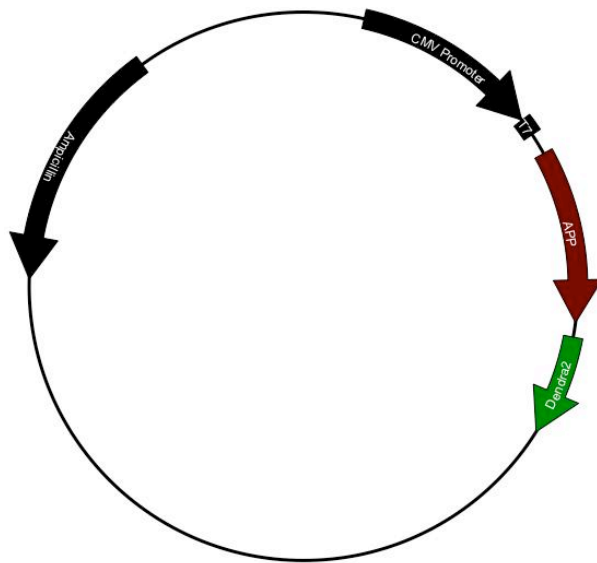
APPENDIX II: CULTURE MEDIA

Defined media for hippocampal neuron cultures

Growth Medium (G2)

Neurobasal™ medium (Invitrogen, 21103-049)   500 ml

- B-27 supplement (Invitrogen, 17504)   10 ml

- 0.5 mM L-glutamine (Invitrogen, 25030-081)   1.27 ml (200 mM solution)

- 0.6% glucose (Sigma, G8769)   6.7 ml (40% solution)

- 1% penicillin/streptomycin (Invitrogen, 15070)   5.0 ml

Plating Medium (G3)

Growth medium (G2) with:

- 25 µM L-glutamate (add fresh)   63.9 µl (200 mM solution)

Minimal Substrate

Poly-D-lysine (Sigma, P6407-5MG) is generally used as minimal substrate for neurons. For coating plastic surfaces used for the culture of neuronal cells, it should be dissolved in boric acid (0.1M Na_2HBO_3, pH 8.1) at a minimum concentration of 10 µg/mL. Coat the plates with this solution for at least 1 hour or overnight at 37°C, then rinse once with distilled water and let dry for 1 hour under the hood to keep the dishes sterile.

Bibliography

Alzheimer, A. (1906). Über einen eigenartigen schweren Erkrankungsprozeß der Hirnrinde. Neurologisches Centralblatt *23*, 1129-1136.

Andersen, O.M., Reiche, J., Schmidt, V., Gotthardt, M., Spoelgen, R., Behlke, J., von Arnim, C.A., Breiderhoff, T., Jansen, P., Wu, X.*, et al.* (2005). Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proc Natl Acad Sci U S A *102*, 13461-13466.

Ando, K., Oishi, M., Takeda, S., Iijima, K., Isohara, T., Nairn, A.C., Kirino, Y., Greengard, P., and Suzuki, T. (1999). Role of phosphorylation of Alzheimer's amyloid precursor protein during neuronal differentiation. J Neurosci *19*, 4421-4427.

Anliker, B., and Muller, U. (2006). The functions of mammalian amyloid precursor protein and related amyloid precursor-like proteins. Neurodegener Dis *3*, 239-246.

Apelt, J., Ach, K., and Schliebs, R. (2003). Aging-related down-regulation of neprilysin, a putative beta-amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of beta-amyloid plaques. Neurosci Lett *339*, 183-186.

Araki, W., Kitaguchi, N., Tokushima, Y., Ishii, K., Aratake, H., Shimohama, S., Nakamura, S., and Kimura, J. (1991). Trophic effect of beta-amyloid precursor protein on cerebral cortical neurons in culture. Biochem Biophys Res Commun *181*, 265-271.

Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T., and Hyman, B.T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology *42*, 631-639.

Baig, S., Joseph, S.A., Tayler, H., Abraham, R., Owen, M.J., Williams, J., Kehoe, P.G., and Love, S. (2010). Distribution and expression of picalm in Alzheimer disease. J Neuropathol Exp Neurol *69*, 1071-1077.

Barghorn, S., Nimmrich, V., Striebinger, A., Krantz, C., Keller, P., Janson, B., Bahr, M., Schmidt, M., Bitner, R., Harlan, J.*, et al.* (2005). Globular amyloid beta-peptide oligomer - a homogenous and stable neuropathological protein in Alzheimer's disease. J Neurochem *95*, 834-847.

Barnes, N.Y., Li, L., Yoshikawa, K., Schwartz, L.M., Oppenheim, R.W., and Milligan, C.E. (1998). Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons. J Neurosci *18*, 5869-5880.

Baulac, S., LaVoie, M.J., Kimberly, W.T., Strahle, J., Wolfe, M.S., Selkoe, D.J., and Xia, W. (2003). Functional gamma-secretase complex assembly in Golgi/trans-Golgi network: interactions among presenilin, nicastrin, Aph1, Pen-2, and gamma-secretase substrates. Neurobiol Dis *14*, 194-204.

Beher, D., Hesse, L., Masters, C.L., and Multhaup, G. (1996). Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. J Biol Chem *271*, 1613-1620.

Bertram, L., Lill, C.M., and Tanzi, R.E. (2010). The genetics of Alzheimer disease: back to the future. Neuron *68*, 270-281.

Bertrand, E., Brouillet, E., Caille, I., Bouillot, C., Cole, G., Prochiantz, A., and Allinquant, B. (2001). A short cytoplasmic domain of the amyloid precursor protein induces apoptosis in vitro and in vivo. Mol Cell Neurosci *18*, 503-511.

Beyreuther, K., Multhaup, G., Simms, G., Pottgiesser, J., Schroeder, W., Martins, R.N., and Masters, C.L. (1986). Discussions in Neuroscience *3*, 68-79.

Bierer, L.M., Hof, P.R., Purohit, D.P., Carlin, L., Schmeidler, J., Davis, K.L., and Perl, D.P. (1995). Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. Arch Neurol *52*, 81-88.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S.*, et al.* (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature *385*, 729-733.

Blasko, I., Beer, R., Bigl, M., Apelt, J., Franz, G., Rudzki, D., Ransmayr, G., Kampfl, A., and Schliebs, R. (2004). Experimental traumatic brain injury in rats stimulates the expression, production and activity of Alzheimer's disease beta-secretase (BACE-1). J Neural Transm *111*, 523-536.

Blessed, G., Tomlinson, B.E., and Roth, M. (1968). The association between quantitative measures of dementia and of senile change in the cerebral grey matter of elderly subjects. Br J Psychiatry *114*, 797-811.

Boller, F., Bick, K., and Duyckaerts, C. (2007). They have shaped Alzheimer disease: the protagonists, well known and less well known. Cortex *43*, 565-569.

Bonifacino, J.S. (2004). The GGA proteins: adaptors on the move. Nat Rev Mol Cell Biol *5*, 23-32.

Borg, J.P., Ooi, J., Levy, E., and Margolis, B. (1996). The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. Mol Cell Biol *16*, 6229-6241.

Bormann, B.J., Knowles, W.J., and Marchesi, V.T. (1989). Synthetic peptides mimic the assembly of transmembrane glycoproteins. J Biol Chem *264*, 4033-4037.

Bouron, A., Mbebi, C., Loeffler, J.P., and De Waard, M. (2004). The beta-amyloid precursor protein controls a store-operated Ca2+ entry in cortical neurons. Eur J Neurosci *20*, 2071-2078.

Braak, H., and Braak, E. (1985). On areas of transition between entorhinal allocortex and temporal isocortex in the human brain. Normal morphology and lamina-specific pathology in Alzheimer's disease. Acta Neuropathol *68*, 325-332.

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol *82*, 239-259.

Braak, H., Braak, E., and Bohl, J. (1993). Staging of Alzheimer-related cortical destruction. Eur Neurol *33*, 403-408.

Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res *35*, 567-576.

Brosnan, C.A., and Voinnet, O. (2009). The long and the short of noncoding RNAs. Curr Opin Cell Biol *21*, 416-425.

Buda, O., Arsene, D., Ceausu, M., Dermengiu, D., and Curca, G.C. (2009). Georges Marinesco and the early research in neuropathology. Neurology *72*, 88-91.

Buee, L., Ding, W., Anderson, J.P., Narindrasorasak, S., Kisilevsky, R., Boyle, N.J., Robakis, N.K., Delacourte, A., Greenberg, B., and Fillit, H.M. (1993a). Binding of vascular heparan sulfate proteoglycan to Alzheimer's amyloid precursor protein is mediated in part by the N-terminal region of A4 peptide. Brain Res *627*, 199-204.

Buee, L., Ding, W., Delacourte, A., and Fillit, H. (1993b). Binding of secreted human neuroblastoma proteoglycans to the Alzheimer's amyloid A4 peptide. Brain Res *601*, 154-163.

Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C., and Glabe, C. (1992). Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J Biol Chem *267*, 546-554.

Burger, C. (2010). Region-specific genetic alterations in the aging hippocampus: implications for cognitive aging. Front Aging Neurosci *2*, 140.

Bush, A.I., Multhaup, G., Moir, R.D., Williamson, T.G., Small, D.H., Rumble, B., Pollwein, P., Beyreuther, K., and Masters, C.L. (1993). A novel zinc(II) binding site

modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease. J Biol Chem *268*, 16109-16112.

Caceres, J., and Brandan, E. (1997). Interaction between Alzheimer's disease beta A4 precursor protein (APP) and the extracellular matrix: evidence for the participation of heparan sulfate proteoglycans. J Cell Biochem *65*, 145-158.

Cai, X.D., Golde, T.E., and Younkin, S.G. (1993). Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science *259*, 514-516.

Cam, J.A., Zerbinatti, C.V., Knisely, J.M., Hecimovic, S., Li, Y., and Bu, G. (2004). The low density lipoprotein receptor-related protein 1B retains beta-amyloid precursor protein at the cell surface and reduces amyloid-beta peptide production. J Biol Chem *279*, 29639-29646.

Cam, J.A., Zerbinatti, C.V., Li, Y., and Bu, G. (2005). Rapid endocytosis of the low density lipoprotein receptor-related protein modulates cell surface distribution and processing of the beta-amyloid precursor protein. J Biol Chem *280*, 15464-15470.

Cao, X., and Sudhof, T. (2001). A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science *293*, 115-120.

Carrasquillo, M.M., Zou, F., Pankratz, V.S., Wilcox, S.L., Ma, L., Walker, L.P., Younkin, S.G., Younkin, C.S., Younkin, L.H., Bisceglio, G.D*., et al.* (2009). Genetic variation in PCDH11X is associated with susceptibility to late-onset Alzheimer's disease. Nat Genet *41*, 192-198.

Castellani, R.J., Lee, H.G., Zhu, X., Perry, G., and Smith, M.A. (2008). Alzheimer disease pathology as a host response. J Neuropathol Exp Neurol *67*, 523-531.

Castellani, R.J., Rolston, R.K., and Smith, M.A. (2010). Alzheimer disease. Dis Mon *56*, 484-546.

Castillo, G.M., Ngo, C., Cummings, J., Wight, T.N., and Snow, A.D. (1997). Perlecan binds to the beta-amyloid proteins (A beta) of Alzheimer's disease, accelerates A beta fibril formation, and maintains A beta fibril stability. J Neurochem *69*, 2452-2465.

Caughey, B., and Lansbury, P.T. (2003). Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. Annu Rev Neurosci *26*, 267-298.

Chapman, P.F., White, G.L., Jones, M.W., Cooper-Blacketer, D., Marshall, V.J., Irizarry, M., Younkin, L., Good, M.A., Bliss, T.V., Hyman, B.T*., et al.* (1999). Impaired

synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci *2*, 271-276.

Chen, C.D., Oh, S.Y., Hinman, J.D., and Abraham, C.R. (2006). Visualization of APP dimerization and APP-Notch2 heterodimerization in living cells using bimolecular fluorescence complementation. J Neurochem *97*, 30-43.

Chouliaras, L., Rutten, B.P., Kenis, G., Peerbooms, O., Visser, P.J., Verhey, F., van Os, J., Steinbusch, H.W., and van den Hove, D.L. (2010). Epigenetic regulation in the pathophysiology of Alzheimer's disease. Prog Neurobiol *90*, 498-510.

Chow, V.W., Mattson, M.P., Wong, P.C., and Gleichmann, M. (2009). An Overview of APP Processing Enzymes and Products. Neuromolecular Med.

Chui, D.H., Dobo, E., Makifuchi, T., Akiyama, H., Kawakatsu, S., Petit, A., Checler, F., Araki, W., Takahashi, K., and Tabira, T. (2001). Apoptotic neurons in Alzheimer's disease frequently show intracellular Abeta42 labeling. J Alzheimers Dis *3*, 231-239.

Cirrito, J.R., Kang, J.E., Lee, J., Stewart, F.R., Verges, D.K., Silverio, L.M., Bu, G., Mennerick, S., and Holtzman, D.M. (2008). Endocytosis is required for synaptic activity-dependent release of amyloid-beta in vivo. Neuron *58*, 42-51.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D.J. (1992). Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature *360*, 672-674.

Cleary, J.P., Walsh, D.M., Hofmeister, J.J., Shankar, G.M., Kuskowski, M.A., Selkoe, D.J., and Ashe, K.H. (2005). Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci *8*, 79-84.

Cook, D.G., Leverenz, J.B., McMillan, P.J., Kulstad, J.J., Ericksen, S., Roth, R.A., Schellenberg, G.D., Jin, L.W., Kovacina, K.S., and Craft, S. (2003). Reduced hippocampal insulin-degrading enzyme in late-onset Alzheimer's disease is associated with the apolipoprotein E-epsilon4 allele. Am J Pathol *162*, 313-319.

Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Jr., Rimmler, J.B., Locke, P.A., Conneally, P.M., Schmader, K.E.*, et al.* (1994). Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet *7*, 180-184.

Costantini, C., Della-Bianca, V., Formaggio, E., Chiamulera, C., Montresor, A., and Rossi, F. (2005). The expression of p75 neurotrophin receptor protects against

the neurotoxicity of soluble oligomers of beta-amyloid. Exp Cell Res *311*, 126-134.

Coulson, E.J. (2006). Does the p75 neurotrophin receptor mediate Abeta-induced toxicity in Alzheimer's disease? J Neurochem *98*, 654-660.

Craig-Schapiro, R., Fagan, A.M., and Holtzman, D.M. (2009). Biomarkers of Alzheimer's disease. Neurobiol Dis *35*, 128-140.

Cras, P., van Harskamp, F., Hendriks, L., Ceuterick, C., van Duijn, C.M., Stefanko, S.Z., Hofman, A., Kros, J.M., Van Broeckhoven, C., and Martin, J.J. (1998). Presenile Alzheimer dementia characterized by amyloid angiopathy and large amyloid core type senile plaques in the APP 692Ala-->Gly mutation. Acta Neuropathol *96*, 253-260.

Dahms, S.O., Hoefgen, S., Roeser, D., Schlott, B., Guhrs, K.H., and Than, M.E. (2010). Structure and biochemical analysis of the heparin-induced E1 dimer of the amyloid precursor protein. Proc Natl Acad Sci U S A *107*, 5381-5386.

Daigle, I., and Li, C. (1993). apl-1, a Caenorhabditis elegans gene encoding a protein related to the human beta-amyloid protein precursor. Proc Natl Acad Sci U S A *90*, 12045-12049.

Davidson, T.J., Harel, S., Arboleda, V.A., Prunell, G.F., Shelanski, M.L., Greene, L.A., and Troy, C.M. (2004). Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation. J Neurosci *24*, 10040-10046.

Dawson, G.R., Seabrook, G.R., Zheng, H., Smith, D.W., Graham, S., O'Dowd, G., Bowery, B.J., Boyce, S., Trumbauer, M.E., Chen, H.Y.*, et al.* (1999). Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. Neuroscience *90*, 1-13.

De Felice, F., Vieira, M., Bomfim, T., Decker, H., Velasco, P., Lambert, M., Viola, K., Zhao, W., Ferreira, S., and Klein, W. (2009). Protection of synapses against Alzheimer's-linked toxins: Insulin signaling prevents the pathogenic binding of A {beta} oligomers. Proc Natl Acad Sci USA.

De Felice, F.G., and Ferreira, S.T. (2002). Beta-amyloid production, aggregation, and clearance as targets for therapy in Alzheimer's disease. Cell Mol Neurobiol *22*, 545-563.

De Felice, F.G., Velasco, P.T., Lambert, M.P., Viola, K., Fernandez, S.J., Ferreira, S.T., and Klein, W.L. (2007). Abeta Oligomers Induce Neuronal Oxidative Stress

through an N-Methyl-D-aspartate Receptor-dependent Mechanism That Is Blocked by the Alzheimer Drug Memantine. J Biol Chem *282*, 11590-11601.

De Jonghe, C., Zehr, C., Yager, D., Prada, C.M., Younkin, S., Hendriks, L., Van Broeckhoven, C., and Eckman, C.B. (1998). Flemish and Dutch mutations in amyloid beta precursor protein have different effects on amyloid beta secretion. Neurobiol Dis *5*, 281-286.

De Strooper, B., and Annaert, W. (2000). Proteolytic processing and cell biological functions of the amyloid precursor protein. J Cell Sci *113 (Pt 11)*, 1857-1870.

Dechant, G., and Barde, Y.A. (2002). The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. Nat Neurosci *5*, 1131-1136.

DeKosky, S.T., Scheff, S.W., and Styren, S.D. (1996). Structural correlates of cognition in dementia: quantification and assessment of synapse change. Neurodegeneration *5*, 417-421.

Delaere, P., Duyckaerts, C., Brion, J.P., Poulain, V., and Hauw, J.J. (1989). Tau, paired helical filaments and amyloid in the neocortex: a morphometric study of 15 cases with graded intellectual status in aging and senile dementia of Alzheimer type. Acta Neuropathol *77*, 645-653.

DeMattos, R.B., Cirrito, J.R., Parsadanian, M., May, P.C., O'Dell, M.A., Taylor, J.W., Harmony, J.A., Aronow, B.J., Bales, K.R., Paul, S.M.*, et al.* (2004). ApoE and clusterin cooperatively suppress Abeta levels and deposition: evidence that ApoE regulates extracellular Abeta metabolism in vivo. Neuron *41*, 193-202.

DeMattos, R.B., O'Dell M, A., Parsadanian, M., Taylor, J.W., Harmony, J.A., Bales, K.R., Paul, S.M., Aronow, B.J., and Holtzman, D.M. (2002). Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A *99*, 10843-10848.

Dickson, D.W., Crystal, H.A., Bevona, C., Honer, W., Vincent, I., and Davies, P. (1995). Correlations of synaptic and pathological markers with cognition of the elderly. Neurobiol Aging *16*, 285-298; discussion 298-304.

Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., de Saint Andrieu, P., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J.*, et al.* (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J Neurochem *76*, 173-181.

Dyrks, T., Dyrks, E., Masters, C.L., and Beyreuther, K. (1993). Amyloidogenicity of rodent and human beta A4 sequences. FEBS Lett *324*, 231-236.

Edbauer, D., Winkler, E., Regula, J.T., Pesold, B., Steiner, H., and Haass, C. (2003). Reconstitution of gamma-secretase activity. Nat Cell Biol *5*, 486-488.

Eggert, S., Midthune, B., Cottrell, B., and Koo, E.H. (2009). Induced dimerization of the amyloid precursor protein leads to decreased amyloid-beta protein production. J Biol Chem *284*, 28943-28952.

Ehehalt, R., Keller, P., Haass, C., Thiele, C., and Simons, K. (2003). Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol *160*, 113-123.

Ehrlich, I., Klein, M., Rumpel, S., and Malinow, R. (2007). PSD-95 is required for activity-driven synapse stabilization. Proc Natl Acad Sci U S A *104*, 4176-4181.

Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D., and Ward, P.J. (1990). Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science *248*, 1122-1124.

Faghihi, M.A., Modarresi, F., Khalil, A.M., Wood, D.E., Sahagan, B.G., Morgan, T.E., Finch, C.E., St Laurent, G., Kenny, P.J., and Wahlestedt, C. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat Med *14*, 723-730.

Faghihi, M.A., Zhang, M., Huang, J., Modarresi, F., Van der Brug, M.P., Nalls, M.A., Cookson, M.R., St-Laurent, G., 3rd, and Wahlestedt, C. (2010). Evidence for natural antisense transcript-mediated inhibition of microRNA function. Genome Biol *11*, R56.

Farrer, L.A., Cupples, L.A., Haines, J.L., Hyman, B., Kukull, W.A., Mayeux, R., Myers, R.H., Pericak-Vance, M.A., Risch, N., and van Duijn, C.M. (1997). Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA *278*, 1349-1356.

Farris, W., Schütz, S.G., Cirrito, J.R., Shankar, G.M., Sun, X., George, A., Leissring, M.A., Walsh, D.M., Qiu, W.Q., Holtzman, D.M.*, et al.* (2007). Loss of neprilysin function promotes amyloid plaque formation and causes cerebral amyloid angiopathy. Am J Pathol *171*, 241-251.

Farzan, M., Schnitzler, C.E., Vasilieva, N., Leung, D., and Choe, H. (2000). BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. Proc Natl Acad Sci U S A *97*, 9712-9717.

Ferreira, S.T., Vieira, M.N., and De Felice, F.G. (2007). Soluble protein oligomers as emerging toxins in Alzheimer's and other amyloid diseases. IUBMB Life *59*, 332-345.

Forstl, H., and Kurz, A. (1999). Clinical features of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci *249*, 288-290.

Fox, N.C., and Freeborough, P.A. (1997). Brain atrophy progression measured from registered serial MRI: validation and application to Alzheimer's disease. J Magn Reson Imaging *7*, 1069-1075.

Fox, N.C., Freeborough, P.A., and Rossor, M.N. (1996). Visualisation and quantification of rates of atrophy in Alzheimer's disease. Lancet *348*, 94-97.

Francis, R., McGrath, G., Zhang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C.*, et al.* (2002). aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell *3*, 85-97.

Freir, D.B., Holscher, C., and Herron, C.E. (2001). Blockade of long-term potentiation by beta-amyloid peptides in the CA1 region of the rat hippocampus in vivo. J Neurophysiol *85*, 708-713.

Frisoni, G.B., Laakso, M.P., Beltramello, A., Geroldi, C., Bianchetti, A., Soininen, H., and Trabucchi, M. (1999). Hippocampal and entorhinal cortex atrophy in frontotemporal dementia and Alzheimer's disease. Neurology *52*, 91-100.

Fukuda, M., Viitala, J., Matteson, J., and Carlsson, S.R. (1988). Cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Comparison of their deduced amino acid sequences. J Biol Chem *263*, 18920-18928.

Fukumoto, H., Cheung, B.S., Hyman, B.T., and Irizarry, M.C. (2002). Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol *59*, 1381-1389.

Furukawa, K., and Mattson, M.P. (1998). Secreted amyloid precursor protein alpha selectively suppresses N-methyl-D-aspartate currents in hippocampal neurons: involvement of cyclic GMP. Neuroscience *83*, 429-438.

Galli, C., Piccini, A., Ciotti, M.T., Castellani, L., Calissano, P., Zaccheo, D., and Tabaton, M. (1998). Increased amyloidogenic secretion in cerebellar granule cells undergoing apoptosis. Proc Natl Acad Sci U S A *95*, 1247-1252.

Galvan, V., Chen, S., Lu, D., Logvinova, A., Goldsmith, P., Koo, E.H., and Bredesen, D.E. (2002). Caspase cleavage of members of the amyloid precursor family of proteins. J Neurochem *82*, 283-294.

Galvan, V., Gorostiza, O.F., Banwait, S., Ataie, M., Logvinova, A.V., Sitaraman, S., Carlson, E., Sagi, S.A., Chevallier, N., Jin, K.*, et al.* (2006). Reversal of Alzheimer's-like pathology and behavior in human APP transgenic mice by mutation of Asp664. Proc Natl Acad Sci USA *103*, 7130-7135.

Gan, W.B., Grutzendler, J., Wong, W.T., Wong, R.O., and Lichtman, J.W. (2000). Multicolor "DiOlistic" labeling of the nervous system using lipophilic dye combinations. Neuron *27*, 219-225.

Garver, T.D., Lehman, R.A., and Billingsley, M.L. (1996). Microtubule assembly competence analysis of freshly-biopsied human tau, dephosphorylated tau, and Alzheimer tau. J Neurosci Res *44*, 12-20.

Gelissen, I.C., Hochgrebe, T., Wilson, M.R., Easterbrook-Smith, S.B., Jessup, W., Dean, R.T., and Brown, A.J. (1998). Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function? Biochem J *331 (Pt 1)*, 231-237.

Gerdes, L.U., Jeune, B., Ranberg, K.A., Nybo, H., and Vaupel, J.W. (2000). Estimation of apolipoprotein E genotype-specific relative mortality risks from the distribution of genotypes in centenarians and middle-aged men: apolipoprotein E gene is a "frailty gene," not a "longevity gene". Genet Epidemiol *19*, 202-210.

Gervais, F.G., Xu, D., Robertson, G.S., Vaillancourt, J.P., Zhu, Y., Huang, J., LeBlanc, A., Smith, D., Rigby, M., Shearman, M.S.*, et al.* (1999). Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. Cell *97*, 395-406.

Ghiso, J., Rostagno, A., Gardella, J.E., Liem, L., Gorevic, P.D., and Frangione, B. (1992). A 109-amino-acid C-terminal fragment of Alzheimer's-disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion. Biochem J *288 (Pt 3)*, 1053-1059.

Ghribi, O. (2006). The role of the endoplasmic reticulum in the accumulation of beta-amyloid peptide in Alzheimer's disease. Curr Mol Med *6*, 119-133.

Glabe, C.G. (2008). Structural classification of toxic amyloid oligomers. J Biol Chem *283*, 29639-29643.

Glenner, G.G., Ein, D., Eanes, E.D., Bladen, H.A., Terry, W., and Page, D.L. (1971a). Creation of "amyloid" fibrils from Bence Jones proteins in vitro. Science *174*, 712-714.

Glenner, G.G., Terry, W., Harada, M., Isersky, C., and Page, D. (1971b). Amyloid fibril proteins: proof of homology with immunoglobulin light chains by sequence analyses. Science *172*, 1150-1151.

Glenner, G.G., and Wong, C.W. (1984). Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem Biophys Res Commun *122*, 1131-1135.

Goodman, Y., and Mattson, M.P. (1994). Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. Exp Neurol *128*, 1-12.

Gorman, P.M., and Chakrabartty, A. (2001). Alzheimer beta-amyloid peptides: structures of amyloid fibrils and alternate aggregation products. Biopolymers *60*, 381-394.

Goutte, C., Hepler, W., Mickey, K.M., and Priess, J.R. (2000). aph-2 encodes a novel extracellular protein required for GLP-1-mediated signaling. Development *127*, 2481-2492.

Gralle, M., Botelho, M.G., and Wouters, F.S. (2009). Neuroprotective secreted amyloid precursor protein acts by disrupting amyloid precursor protein dimers. J Biol Chem *284*, 15016-15025.

Gralle, M., and Ferreira, S.T. (2007). Structure and functions of the human amyloid precursor protein: the whole is more than the sum of its parts. Prog Neurobiol *82*, 11-32.

Gralle, M., Oliveira, C.L.P., Guerreiro, L.H., McKinstry, W.J., Galatis, D., Masters, C.L., Cappai, R., Parker, M.W., Ramos, C.H.I., Torriani, I.*, et al.* (2006). Solution conformation and heparin-induced dimerization of the full-length extracellular domain of the human amyloid precursor protein. J Mol Biol *357*, 493-508.

Greenfield, J.P., Tsai, J., Gouras, G.K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S.S., Greengard, P., and Xu, H. (1999). Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides. Proc Natl Acad Sci U S A *96*, 742-747.

Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S., and Wisniewski, H.M. (1986). Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem *261*, 6084-6089.

Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takio, K., and Ihara, Y. (2001). Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch. J Biol Chem *276*, 35235-35238.

Gunn, A.P., Masters, C.L., and Cherny, R.A. (2010). Pyroglutamate-Abeta: Role in the natural history of Alzheimer's disease. Int J Biochem Cell Biol *42*, 1915-1918.

Guo, Q., Xie, J., Chang, X., and Du, H. (2001). Prostate apoptosis response-4 enhances secretion of amyloid beta peptide 1-42 in human neuroblastoma IMR-32 cells by a caspase-dependent pathway. J Biol Chem *276*, 16040-16044.

Gurskaya, N.G., Verkhusha, V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F., Lukyanov, S., and Lukyanov, K.A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol *24*, 461-465.

Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B.*, et al.* (1992). Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature *359*, 322-325.

Haass, C., and Selkoe, D.J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol *8*, 101-112.

Hansson, C., Popescu, B., Laudon, H., Cedazo-Minguez, A., Popescu, L., Winblad, B., and Ankarcrona, M. (2006). Caspase cleaved presenilin-1 is part of active gamma-secretase complexes. J Neurochem *97*, 356-364.

Harada, J., and Sugimoto, M. (1999). Activation of caspase-3 in beta-amyloid-induced apoptosis of cultured rat cortical neurons. Brain Res *842*, 311-323.

Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353-356.

Harel, A., Wu, F., Mattson, M.P., Morris, C.M., and Yao, P.J. (2008). Evidence for CALM in directing VAMP2 trafficking. Traffic *9*, 417-429.

Harigaya, Y., Saido, T.C., Eckman, C.B., Prada, C.M., Shoji, M., and Younkin, S.G. (2000). Amyloid beta protein starting pyroglutamate at position 3 is a major component of the amyloid deposits in the Alzheimer's disease brain. Biochem Biophys Res Commun *276*, 422-427.

Harold, D., Abraham, R., Hollingworth, P., Sims, R., Gerrish, A., Hamshere, M.L., Pahwa, J.S., Moskvina, V., Dowzell, K., Williams, A.*, et al.* (2009). Genome-wide

association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet *41*, 1088-1093.

Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vasquez, S., Vassilev, P.M., Teplow, D.B., and Selkoe, D.J. (1999). Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J Neurosci *19*, 8876-8884.

He, X., Chang, W.P., Koelsch, G., and Tang, J. (2002). Memapsin 2 (beta-secretase) cytosolic domain binds to the VHS domains of GGA1 and GGA2: implications on the endocytosis mechanism of memapsin 2. FEBS Lett *524*, 183-187.

He, X., Li, F., Chang, W.P., and Tang, J. (2005). GGA proteins mediate the recycling pathway of memapsin 2 (BACE). J Biol Chem *280*, 11696-11703.

He, X., Zhu, G., Koelsch, G., Rodgers, K.K., Zhang, X.C., and Tang, J. (2003). Biochemical and structural characterization of the interaction of memapsin 2 (beta-secretase) cytosolic domain with the VHS domain of GGA proteins. Biochemistry *42*, 12174-12180.

Heber, S., Herms, J., Gajic, V., Hainfellner, J., Aguzzi, A., Rulicke, T., von Kretzschmar, H., von Koch, C., Sisodia, S., Tremml, P.*, et al.* (2000). Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci *20*, 7951-7963.

Hebert, L.E., Scherr, P.A., Bienias, J.L., Bennett, D.A., and Evans, D.A. (2003). Alzheimer disease in the US population: prevalence estimates using the 2000 census. Arch Neurol *60*, 1119-1122.

Hébert, S., Horré, K., Nicolaï, L., Papadopoulou, A., Mandemakers, W., Silahtaroglu, A., Kauppinen, S., Delacourte, A., and De Strooper, B. (2008). Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/{beta}-secretase expression. Proc Natl Acad Sci USA.

Hendriks, L., van Duijn, C.M., Cras, P., Cruts, M., Van Hul, W., van Harskamp, F., Warren, A., McInnis, M.G., Antonarakis, S.E., Martin, J.J.*, et al.* (1992). Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. Nat Genet *1*, 218-221.

Herms, J., Anliker, B., Heber, S., Ring, S., Fuhrmann, M., Kretzschmar, H., Sisodia, S., and Muller, U. (2004). Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. EMBO J *23*, 4106-4115.

Heron, M., Hoyert, D.L., Murphy, S.L., Xu, J., Kochanek, K.D., and Tejada-Vera, B. (2009). Deaths: final data for 2006. Natl Vital Stat Rep *57*, 1-134.

Herzog, V., Kirfel, G., Siemes, C., and Schmitz, A. (2004). Biological roles of APP in the epidermis. Eur J Cell Biol *83*, 613-624.

Hesse, L., Beher, D., Masters, C.L., and Multhaup, G. (1994). The beta A4 amyloid precursor protein binding to copper. FEBS Lett *349*, 109-116.

Hilbich, C., Monning, U., Grund, C., Masters, C.L., and Beyreuther, K. (1993). Amyloid-like properties of peptides flanking the epitope of amyloid precursor protein-specific monoclonal antibody 22C11. J Biol Chem *268*, 26571-26577.

Hoffmann, J., Twiesselmann, C., Kummer, M.P., Romagnoli, P., and Herzog, V. (2000). A possible role for the Alzheimer amyloid precursor protein in the regulation of epidermal basal cell proliferation. Eur J Cell Biol *79*, 905-914.

Holsinger, R.M., McLean, C.A., Beyreuther, K., Masters, C.L., and Evin, G. (2002). Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. Ann Neurol *51*, 783-786.

Homayouni, R., Rice, D.S., Sheldon, M., and Curran, T. (1999). Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. J Neurosci *19*, 7507-7515.

Hornsten, A., Lieberthal, J., Fadia, S., Malins, R., Ha, L., Xu, X., Daigle, I., Markowitz, M., O'Connor, G., Plasterk, R.*, et al.* (2007). APL-1, a Caenorhabditis elegans protein related to the human beta-amyloid precursor protein, is essential for viability. Proc Natl Acad Sci U S A *104*, 1971-1976.

Hoyer, S. (1994). Age as risk factor for sporadic dementia of the Alzheimer type? Ann N Y Acad Sci *719*, 248-256.

Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. Proc Natl Acad Sci USA *96*, 3228-3233.

Hu, Y., and Fortini, M.E. (2003). Different cofactor activities in gamma-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex. J Cell Biol *161*, 685-690.

Huse, J.T., Byant, D., Yang, Y., Pijak, D.S., D'Souza, I., Lah, J.J., Lee, V.M., Doms, R.W., and Cook, D.G. (2003). Endoproteolysis of beta-secretase (beta-site amyloid precursor protein-cleaving enzyme) within its catalytic domain. A potential mechanism for regulation. J Biol Chem *278*, 17141-17149.

Huse, J.T., Pijak, D.S., Leslie, G.J., Lee, V.M., and Doms, R.W. (2000). Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. J Biol Chem *275*, 33729-33737.

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M*., et al.* (1999). Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol Cell Neurosci *14*, 419-427.

Hussain, I., Powell, D.J., Howlett, D.R., Chapman, G.A., Gilmour, L., Murdock, P.R., Tew, D.G., Meek, T.D., Chapman, C., Schneider, K*., et al.* (2000). ASP1 (BACE2) cleaves the amyloid precursor protein at the beta-secretase site. Mol Cell Neurosci *16*, 609-619.

Hyman, B.T., Van Hoesen, G.W., Beyreuther, K., and Masters, C.L. (1989). A4 amyloid protein immunoreactivity is present in Alzheimer's disease neurofibrillary tangles. Neurosci Lett *101*, 352-355.

Iacoangeli, A., Bianchi, R., and Tiedge, H. (2010). Regulatory RNAs in brain function and disorders. Brain Res *1338*, 36-47.

Iijima, K., Ando, K., Takeda, S., Satoh, Y., Seki, T., Itohara, S., Greengard, P., Kirino, Y., Nairn, A.C., and Suzuki, T. (2000). Neuron-specific phosphorylation of Alzheimer's beta-amyloid precursor protein by cyclin-dependent kinase 5. J Neurochem *75*, 1085-1091.

Ikeda, S., Allsop, D., and Glenner, G.G. (1989). Morphology and distribution of plaque and related deposits in the brains of Alzheimer's disease and control cases. An immunohistochemical study using amyloid beta-protein antibody. Lab Invest *60*, 113-122.

Ingelsson, M., Fukumoto, H., Newell, K.L., Growdon, J.H., Hedley-Whyte, E.T., Frosch, M.P., Albert, M.S., Hyman, B.T., and Irizarry, M.C. (2004). Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. Neurology *62*, 925-931.

Ionita-Laza, I., McQueen, M.B., Laird, N.M., and Lange, C. (2007). Genomewide weighted hypothesis testing in family-based association studies, with an application to a 100K scan. Am J Hum Genet *81*, 607-614.

Isacson, O., Seo, H., Lin, L., Albeck, D., and Granholm, A.C. (2002). Alzheimer's disease and Down's syndrome: roles of APP, trophic factors and ACh. Trends Neurosci *25*, 79-84.

Ivanov, A., Esclapez, M., and Ferhat, L. (2009). Role of drebrin A in dendritic spine plasticity and synaptic function: Implications in neurological disorders. Commun Integr Biol *2*, 268-270.

Jack, C.R., Jr., Knopman, D.S., Jagust, W.J., Shaw, L.M., Aisen, P.S., Weiner, M.W., Petersen, R.C., and Trojanowski, J.Q. (2010). Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol *9*, 119-128.

Jacobsen, K.T., and Iverfeldt, K. (2009). Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. Cell Mol Life Sci *66*, 2299-2318.

Jarrett, J.T., Berger, E.P., and Lansbury, P.T., Jr. (1993). The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry *32*, 4693-4697.

Jellinger, K.A. (2004). Head injury and dementia. Curr Opin Neurol *17*, 719-723.

Jick, H., Zornberg, G.L., Jick, S.S., Seshadri, S., and Drachman, D.A. (2000). Statins and the risk of dementia. Lancet *356*, 1627-1631.

Jin, L.W., Ninomiya, H., Roch, J.M., Schubert, D., Masliah, E., Otero, D.A., and Saitoh, T. (1994). Peptides containing the RERMS sequence of amyloid beta/A4 protein precursor bind cell surface and promote neurite extension. J Neurosci *14*, 5461-5470.

Kaden, D., Munter, L.M., Joshi, M., Treiber, C., Weise, C., Bethge, T., Voigt, P., Schaefer, M., Beyermann, M., Reif, B.*, et al.* (2008). Homophilic interactions of the amyloid precursor protein (APP) ectodomain are regulated by the loop region and affect beta-secretase cleavage of APP. J Biol Chem *283*, 7271-7279.

Kaden, D., Voigt, P., Munter, L., Bobowski, K., Schaefer, M., and Multhaup, G. (2009). Subcellular localization and dimerization of APLP1 are strikingly different from APP and APLP2. J Cell Sci.

Kakuda, N., Funamoto, S., Yagishita, S., Takami, M., Osawa, S., Dohmae, N., and Ihara, Y. (2006). Equimolar production of amyloid beta-protein and amyloid precursor protein intracellular domain from beta-carboxyl-terminal fragment by gamma-secretase. J Biol Chem *281*, 14776-14786.

Kalaria, R.N. (1993). The immunopathology of Alzheimer's disease and some related disorders. Brain Pathol *3*, 333-347.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S., and Malinow, R. (2003). APP processing and synaptic function. Neuron *37*, 925-937.

Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature *325*, 733-736.

Kang, J., and Muller-Hill, B. (1990). Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4(695) mRNA is predominantly produced in rat and human brain. Biochem Biophys Res Commun *166*, 1192-1200.

Katzman, R. (1986). Alzheimer's disease. N Engl J Med *314*, 964-973.

Kawahara, M., and Kuroda, Y. (2000). Molecular mechanism of neurodegeneration induced by Alzheimer's beta-amyloid protein: channel formation and disruption of calcium homeostasis. Brain Res Bull *53*, 389-397.

Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science *300*, 486-489.

Khalifa, N.B., Van Hees, J., Tasiaux, B., Huysseune, S., Smith, S.O., Constantinescu, S.N., Octave, J.N., and Kienlen-Campard, P. (2010). What is the role of amyloid precursor protein dimerization? Cell Adh Migr *4*, 268-272.

Khera, R., and Das, N. (2009). Complement Receptor 1: disease associations and therapeutic implications. Mol Immunol *46*, 761-772.

Kidd, M. (1963). Paired helical filaments in electron microscopy of Alzheimer's disease. Nature *197*, 192-193.

Kidd, M. (1964). Alzheimer's Disease--an Electron Microscopical Study. Brain *87*, 307-320.

Kienlen-Campard, P., Tasiaux, B., Van Hees, J., Li, M., Huysseune, S., Sato, T., Fei, J.Z., Aimoto, S., Courtoy, P.J., Smith, S.O.*, et al.* (2008). Amyloidogenic processing but not amyloid precursor protein (APP) intracellular C-terminal domain production requires a precisely oriented APP dimer assembled by transmembrane GXXXG motifs. J Biol Chem *283*, 7733-7744.

Kim, H.-S., Kim, E.-M., Lee, J.-P., Park, C.H., Kim, S., Seo, J.-H., Chang, K.-A., Yu, E., Jeong, S.-J., Chong, Y.H.*, et al.* (2003). C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. FASEB J *17*, 1951-1953.

Kim, J., Basak, J.M., and Holtzman, D.M. (2009). The role of apolipoprotein E in Alzheimer's disease. Neuron *63*, 287-303.

Kimberly, W.T., Zheng, J.B., Town, T., Flavell, R.A., and Selkoe, D.J. (2005). Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. J Neurosci *25*, 5533-5543.

Kirschner, D.A., Abraham, C., and Selkoe, D.J. (1986). X-ray diffraction from intraneuronal paired helical filaments and extraneuronal amyloid fibers in Alzheimer disease indicates cross-beta conformation. Proc Natl Acad Sci U S A *83*, 503-507.

Kisselev, A.F., and Goldberg, A.L. (2001). Proteasome inhibitors: from research tools to drug candidates. Chem Biol *8*, 739-758.

Klein, W.L., Krafft, G.A., and Finch, C.E. (2001). Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci *24*, 219-224.

Klein, W.L., Stine, W.B., Jr., and Teplow, D.B. (2004). Small assemblies of unmodified amyloid beta-protein are the proximate neurotoxin in Alzheimer's disease. Neurobiol Aging *25*, 569-580.

Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D.P., Bergstrom, M., Savitcheva, I., Huang, G.F., Estrada, S.*, et al.* (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann Neurol *55*, 306-319.

Koh, Y.H., von Arnim, C.A.F., Hyman, B.T., Tanzi, R.E., and Tesco, G. (2005). BACE is degraded via the lysosomal pathway. J Biol Chem *280*, 32499-32504.

Koike, H., Tomioka, S., Sorimachi, H., Saido, T.C., Maruyama, K., Okuyama, A., Fujisawa-Sehara, A., Ohno, S., Suzuki, K., and Ishiura, S. (1999). Membrane-anchored metalloprotease MDC9 has an alpha-secretase activity responsible for processing the amyloid precursor protein. Biochem J *343 Pt 2*, 371-375.

Kojro, E., Gimpl, G., Lammich, S., Marz, W., and Fahrenholz, F. (2001). Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha -secretase ADAM 10. Proc Natl Acad Sci U S A *98*, 5815-5820.

Koo, E.H., Sisodia, S.S., Archer, D.R., Martin, L.J., Weidemann, A., Beyreuther, K., Fischer, P., Masters, C.L., and Price, D.L. (1990). Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. Proc Natl Acad Sci U S A *87*, 1561-1565.

Koo, E.H., and Squazzo, S.L. (1994). Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J Biol Chem *269*, 17386-17389.

Koo, E.H., Squazzo, S.L., Selkoe, D.J., and Koo, C.H. (1996). Trafficking of cell-surface amyloid beta-protein precursor. I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody. J Cell Sci *109 (Pt 5)*, 991-998.

Korenberg, J.R., Pulst, S.M., Neve, R.L., and West, R. (1989). The Alzheimer amyloid precursor protein maps to human chromosome 21 bands q21.105-q21.05. Genomics *5*, 124-127.

Kosik, K.S., Joachim, C.L., and Selkoe, D.J. (1986). Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci U S A *83*, 4044-4048.

Kowalik-Jankowska, T., Ruta-Dolejsz, M., Wisniewska, K., Lankiewicz, L., and Kozlowski, H. (2002). Possible involvement of copper(II) in Alzheimer disease. Environ Health Perspect *110 Suppl 5*, 869-870.

Kozlowski, L., Stoklosa, T., Omura, S., Wojcik, C., Wojtukiewicz, M.Z., Worowski, K., and Ostrowska, H. (2001). Lactacystin inhibits cathepsin A activity in melanoma cell lines. Tumour Biol *22*, 211-215.

Krause, D.L., and Muller, N. (2010). Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer's disease. Int J Alzheimers Dis *2010*.

Kuentzel, S.L., Ali, S.M., Altman, R.A., Greenberg, B.D., and Raub, T.J. (1993). The Alzheimer beta-amyloid protein precursor/protease nexin-II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells. Biochem J *295 (Pt 2)*, 367-378.

Kumar-Singh, S., Dewachter, I., Moechars, D., Lubke, U., De Jonghe, C., Ceuterick, C., Checler, F., Naidu, A., Cordell, B., Cras, P.*, et al.* (2000). Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. Neurobiol Dis *7*, 9-22.

Kurochkin, I.V., and Goto, S. (1994). Alzheimer's beta-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. FEBS Lett *345*, 33-37.

Kwak, Y.D., Brannen, C.L., Qu, T., Kim, H.M., Dong, X., Soba, P., Majumdar, A., Kaplan, A., Beyreuther, K., and Sugaya, K. (2006). Amyloid precursor protein regulates differentiation of human neural stem cells. Stem Cells Dev *15*, 381-389.

LaFerla, F.M., Green, K.N., and Oddo, S. (2007). Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci *8*, 499-509.

Lai, A., Sisodia, S.S., and Trowbridge, I.S. (1995). Characterization of sorting signals in the beta-amyloid precursor protein cytoplasmic domain. J Biol Chem *270*, 3565-3573.

Laird, F.M., Cai, H., Savonenko, A.V., Farah, M.H., He, K., Melnikova, T., Wen, H., Chiang, H.C., Xu, G., Koliatsos, V.E.*, et al.* (2005). BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J Neurosci *25*, 11693-11709.

Lambert, J.C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., Combarros, O., Zelenika, D., Bullido, M.J., Tavernier, B*., et al.* (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet *41*, 1094-1099.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L*., et al.* (1998). Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A *95*, 6448-6453.

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., and Fahrenholz, F. (1999). Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A *96*, 3922-3927.

Lannfelt, L., Basun, H., Vigo-Pelfrey, C., Wahlund, L.O., Winblad, B., Lieberburg, I., and Schenk, D. (1995). Amyloid beta-peptide in cerebrospinal fluid in individuals with the Swedish Alzheimer amyloid precursor protein mutation. Neurosci Lett *199*, 203-206.

Lauren, J., Gimbel, D.A., Nygaard, H.B., Gilbert, J.W., and Strittmatter, S.M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature *457*, 1128-1132.

LaVoie, M.J., Fraering, P.C., Ostaszewski, B.L., Ye, W., Kimberly, W.T., Wolfe, M.S., and Selkoe, D.J. (2003). Assembly of the gamma-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin. J Biol Chem *278*, 37213-37222.

Lazarov, O., Lee, M., Peterson, D.A., and Sisodia, S.S. (2002). Evidence that synaptically released beta-amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. J Neurosci *22*, 9785-9793.

LeBlanc, A. (1995). Increased production of 4 kDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. J Neurosci *15*, 7837-7846.

Lee, J., Retamal, C., Cuitino, L., Caruano-Yzermans, A., Shin, J.E., van Kerkhof, P., Marzolo, M.P., and Bu, G. (2008a). Adaptor protein sorting nexin 17 regulates amyloid precursor protein trafficking and processing in the early endosomes. J Biol Chem *283*, 11501-11508.

Lee, J.H., Barral, S., and Reitz, C. (2008b). The neuronal sortilin-related receptor gene SORL1 and late-onset Alzheimer's disease. Curr Neurol Neurosci Rep *8*, 384-391.

Lee, M.-S.S., Kao, S.-C.C., Lemere, C.A., Xia, W., Tseng, H.-C.C., Zhou, Y., Neve, R., Ahlijanian, M.K., and Tsai, L.-H.H. (2003). APP processing is regulated by cytoplasmic phosphorylation. J Cell Biol *163*, 83-95.

Lee, V.M., Balin, B.J., Otvos, L., Jr., and Trojanowski, J.Q. (1991). A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. Science *251*, 675-678.

Lemmon, M.A., Treutlein, H.R., Adams, P.D., Brunger, A.T., and Engelman, D.M. (1994). A dimerization motif for transmembrane alpha-helices. Nat Struct Biol *1*, 157-163.

Lenkkeri, U., Kestila, M., Lamerdin, J., McCready, P., Adamson, A., Olsen, A., and Tryggvason, K. (1998). Structure of the human amyloid-precursor-like protein gene APLP1 at 19q13.1. Hum Genet *102*, 192-196.

Lesné, S., Koh, M.T., Kotilinek, L., Kayed, R., Glabe, C.G., Yang, A., Gallagher, M., and Ashe, K.H. (2006). A specific amyloid-beta protein assembly in the brain impairs memory. Nature *440*, 352-357.

Lewczuk, P., Kornhuber, J., Vanderstichele, H., Vanmechelen, E., Esselmann, H., Bibl, M., Wolf, S., Otto, M., Reulbach, U., Kolsch, H.*, et al.* (2008). Multiplexed quantification of dementia biomarkers in the CSF of patients with early dementias and MCI: a multicenter study. Neurobiol Aging *29*, 812-818.

Leyssen, M., Ayaz, D., Hebert, S.S., Reeve, S., De Strooper, B., and Hassan, B.A. (2005). Amyloid precursor protein promotes post-developmental neurite arborization in the Drosophila brain. EMBO J *24*, 2944-2955.

Li, R., Lindholm, K., Yang, L.B., Yue, X., Citron, M., Yan, R., Beach, T., Sue, L., Sabbagh, M., Cai, H.*, et al.* (2004). Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. Proc Natl Acad Sci U S A *101*, 3632-3637.

Liston, P., Fong, W.G., and Korneluk, R.G. (2003). The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene *22*, 8568-8580.

Liu, S.T., Howlett, G., and Barrow, C.J. (1999). Histidine-13 is a crucial residue in the zinc ion-induced aggregation of the A beta peptide of Alzheimer's disease. Biochemistry *38*, 9373-9378.

Liu, W., Crocker, E., Zhang, W., Elliott, J.I., Luy, B., Li, H., Aimoto, S., and Smith, S.O. (2005). Structural role of glycine in amyloid fibrils formed from transmembrane alpha-helices. Biochemistry *44*, 3591-3597.

Lorent, K., Overbergh, L., Moechars, D., De Strooper, B., Van Leuven, F., and Van den Berghe, H. (1995). Expression in mouse embryos and in adult mouse brain of three members of the amyloid precursor protein family, of the alpha-2-macroglobulin receptor/low density lipoprotein receptor-related protein and of its ligands apolipoprotein E, lipoprotein lipase, alpha-2-macroglobulin and the 40,000 molecular weight receptor-associated protein. Neuroscience *65*, 1009-1025.

Lorenzo, A., Yuan, M., Zhang, Z., Paganetti, P.A., Sturchler-Pierrat, C., Staufenbiel, M., Mautino, J., Vigo, F.S., Sommer, B., and Yankner, B.A. (2000). Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. Nat Neurosci *3*, 460-464.

Lu, D.C., Rabizadeh, S., Chandra, S., Shayya, R.F., Ellerby, L.M., Ye, X., Salvesen, G.S., Koo, E.H., and Bredesen, D.E. (2000). A second cytotoxic proteolytic peptide derived from amyloid beta-protein precursor. Nat Med *6*, 397-404.

Lu, D.C., Shaked, G.M., Masliah, E., Bredesen, D.E., and Koo, E.H. (2003a). Amyloid beta protein toxicity mediated by the formation of amyloid-beta protein precursor complexes. Ann Neurol *54*, 781-789.

Lu, D.C., Soriano, S., Bredesen, D.E., and Koo, E.H. (2003b). Caspase cleavage of the amyloid precursor protein modulates amyloid beta-protein toxicity. J Neurochem *87*, 733-741.

Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E., and Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol *155*, 853-862.

Luo, L.Q., Martin-Morris, L.E., and White, K. (1990). Identification, secretion, and neural expression of APPL, a Drosophila protein similar to human amyloid protein precursor. J Neurosci *10*, 3849-3861.

Magdesian, M.H., Carvalho, M.M., Mendes, F.A., Saraiva, L.M., Juliano, M.A., Juliano, L., Garcia-Abreu, J., and Ferreira, S.T. (2008). Amyloid-beta binds to the extracellular cysteine-rich domain of Frizzled and inhibits Wnt/beta-catenin signaling. J Biol Chem *283*, 9359-9368.

Mann, D.M., and Jones, D. (1990). Deposition of amyloid (A4) protein within the brains of persons with dementing disorders other than Alzheimer's disease and Down's syndrome. Neurosci Lett *109*, 68-75.

Marchesi, V.T. (2005). An alternative interpretation of the amyloid Abeta hypothesis with regard to the pathogenesis of Alzheimer's disease. Proc Natl Acad Sci USA *102*, 9093-9098.

Marin, N., Romero, B., Bosch-Morell, F., Llansola, M., Felipo, V., Roma, J., and Romero, F.J. (2000). Beta-amyloid-induced activation of caspase-3 in primary cultures of rat neurons. Mech Ageing Dev *119*, 63-67.

Marks, N., and Berg, M.J. (2010). BACE and gamma-secretase characterization and their sorting as therapeutic targets to reduce amyloidogenesis. Neurochem Res *35*, 181-210.

Masliah, E., Mallory, M., Hansen, L., DeTeresa, R., and Terry, R.D. (1993). Quantitative synaptic alterations in the human neocortex during normal aging. Neurology *43*, 192-197.

Masliah, E., and Terry, R. (1993). The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. Brain Pathol *3*, 77-85.

Masliah, E., Terry, R.D., DeTeresa, R.M., and Hansen, L.A. (1989). Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. Neurosci Lett *103*, 234-239.

Maslow, K. (2010). 2010 Alzheimer's disease facts and figures. Alzheimers Dement *6*, 158-194.

Masters, C.L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R.N., and Beyreuther, K. (1985a). Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. EMBO J *4*, 2757-2763.

Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. (1985b). Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A *82*, 4245-4249.

Matrone, C., Ciotti, M., Mercanti, D., Marolda, R., and Calissano, P. (2008a). NGF and BDNF signaling control amyloidogenic route and A{beta} production in hippocampal neurons. Proc Natl Acad Sci USA.

Matrone, C., Di Luzio, A., Meli, G., D'Aguanno, S., Severini, C., Ciotti, M.T., Cattaneo, A., and Calissano, P. (2008b). Activation of the amyloidogenic route by NGF deprivation induces apoptotic death in PC12 cells. J Alzheimers Dis *13*, 81-96.

Matsuda, S., Yasukawa, T., Homma, Y., Ito, Y., Niikura, T., Hiraki, T., Hirai, S., Ohno, S., Kita, Y., Kawasumi, M.*, et al.* (2001). c-Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK. J Neurosci *21*, 6597-6607.

Mattson, M.P., Cheng, B., Culwell, A.R., Esch, F.S., Lieberburg, I., and Rydel, R.E. (1993). Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the beta-amyloid precursor protein. Neuron *10*, 243-254.

Maurer, K., Volk, S., and Gerbaldo, H. (1997). Auguste D and Alzheimer's disease. Lancet *349*, 1546-1549.

Maynard, C.J., Bush, A.I., Masters, C.L., Cappai, R., and Li, Q.X. (2005). Metals and amyloid-beta in Alzheimer's disease. Int J Exp Pathol *86*, 147-159.

Mbebi, C., See, V., Mercken, L., Pradier, L., Muller, U., and Loeffler, J.P. (2002). Amyloid precursor protein family-induced neuronal death is mediated by impairment of the neuroprotective calcium/calmodulin protein kinase IV-dependent signaling pathway. J Biol Chem *277*, 20979-20990.

McStay, G.P., Salvesen, G.S., and Green, D.R. (2008). Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. Cell Death Differ *15*, 322-331.

Mehler, M.F., and Mattick, J.S. (2007). Noncoding RNAs and RNA editing in brain development, functional diversification, and neurological disease. Physiol Rev *87*, 799-823.

Menendez-Gonzalez, M., Perez-Pinera, P., Martinez-Rivera, M., Calatayud, M.T., and Blazquez Menes, B. (2005). APP processing and the APP-KPI domain involvement in the amyloid cascade. Neurodegener Dis *2*, 277-283.

Meziane, H., Dodart, J.C., Mathis, C., Little, S., Clemens, J., Paul, S.M., and Ungerer, A. (1998). Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnestic mice. Proc Natl Acad Sci U S A *95*, 12683-12688.

Miller, C.C., McLoughlin, D.M., Lau, K.F, Tennant, M.E., and Rogelj, B. (2006). The X11 proteins, Abeta production and Alzheimer's disease. Trends Neurosci *29*, 280-285.

Milward, E.A., Papadopoulos, R., Fuller, S.J., Moir, R.D., Small, D., Beyreuther, K., and Masters, C.L. (1992). The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. Neuron *9*, 129-137.

Miners, J.S., Baig, S., Palmer, J., Palmer, L.E., Kehoe, P.G., and Love, S. (2008). Abeta-degrading enzymes in Alzheimer's disease. Brain Pathol *18*, 240-252.

Mizui, T., Takahashi, H., Sekino, Y., and Shirao, T. (2005). Overexpression of drebrin A in immature neurons induces the accumulation of F-actin and PSD-95 into dendritic filopodia, and the formation of large abnormal protrusions. Mol Cell Neurosci *30*, 630-638.

Moechars, D., Dewachter, I., Lorent, K., Reverse, D., Baekelandt, V., Naidu, A., Tesseur, I., Spittaels, K., Haute, C.V., Checler, F*., et al.* (1999). Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. J Biol Chem *274*, 6483-6492.

Morais, V.A., Crystal, A.S., Pijak, D.S., Carlin, D., Costa, J., Lee, V.M., and Doms, R.W. (2003). The transmembrane domain region of nicastrin mediates direct interactions with APH-1 and the gamma-secretase complex. J Biol Chem *278*, 43284-43291.

Moss, M.L., Jin, S.L., Milla, M.E., Bickett, D.M., Burkhart, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsbury, J.R., Hassler, D*., et al.* (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature *385*, 733-736.

Mousnier, A., Kubat, N., Massias-Simon, A., Segeral, E., Rain, J.C., Benarous, R., Emiliani, S., and Dargemont, C. (2007). von Hippel Lindau binding protein 1-mediated degradation of integrase affects HIV-1 gene expression at a postintegration step. Proc Natl Acad Sci U S A *104*, 13615-13620.

Moya, K.L., Benowitz, L.I., Schneider, G.E., and Allinquant, B. (1994). The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis. Dev Biol *161*, 597-603.

Mu, F.T., Callaghan, J.M., Steele-Mortimer, O., Stenmark, H., Parton, R.G., Campbell, P.L., McCluskey, J., Yeo, J.P., Tock, E.P., and Toh, B.H. (1995). EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. J Biol Chem *270*, 13503-13511.

Muller, U., Cristina, N., Li, Z.W., Wolfer, D.P., Lipp, H.P., Rulicke, T., Brandner, S., Aguzzi, A., and Weissmann, C. (1994). Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell *79*, 755-765.

Munter, L.-M., Botev, A., Richter, L., Hildebrand, P.W., Althoff, V., Weise, C., Kaden, D., and Multhaup, G. (2010). Aberrant APP processing in hereditary forms of Alzheimer disease caused by APP-FAD mutations can be rescued by mutations in the APP GxxxG motif. J Biol Chem.

Munter, L.-M., Voigt, P., Harmeier, A., Kaden, D., Gottschalk, K.E., Weise, C., Pipkorn, R., Schaefer, M., Langosch, D., and Multhaup, G. (2007). GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of Abeta42. EMBO J *26*, 1702-1712.

Muresan, Z., and Muresan, V. (2005a). c-Jun NH2-terminal kinase-interacting protein-3 facilitates phosphorylation and controls localization of amyloid-beta precursor protein. J Neurosci *25*, 3741-3751.

Muresan, Z., and Muresan, V. (2005b). Coordinated transport of phosphorylated amyloid-beta precursor protein and c-Jun NH2-terminal kinase-interacting protein-1. J Cell Biol *171*, 615-625.

Narindrasorasak, S., Lowery, D., Gonzalez-DeWhitt, P., Poorman, R.A., Greenberg, B., and Kisilevsky, R. (1991). High affinity interactions between the Alzheimer's beta-amyloid precursor proteins and the basement membrane form of heparan sulfate proteoglycan. J Biol Chem *266*, 12878-12883.

Narindrasorasak, S., Lowery, D.E., Altman, R.A., Gonzalez-DeWhitt, P.A., Greenberg, B.D., and Kisilevsky, R. (1992). Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins. Lab Invest *67*, 643-652.

Naslund, J., Schierhorn, A., Hellman, U., Lannfelt, L., Roses, A.D., Tjernberg, L.O., Silberring, J., Gandy, S.E., Winblad, B., Greengard, P*., et al.* (1994). Relative abundance of Alzheimer A beta amyloid peptide variants in Alzheimer disease and normal aging. Proc Natl Acad Sci U S A *91*, 8378-8382.

Nathalie, P., and Jean-Noel, O. (2008). Processing of amyloid precursor protein and amyloid peptide neurotoxicity. Curr Alzheimer Res *5*, 92-99.

Nguyen, Galvan, Huang, Banwait, Tang, Zhang, and Bredesen (2007). Signal transduction in Alzheimer disease: p21-activated kinase signaling requires C-terminal cleavage of APP at Asp664. J Neurochem.

Niimura, M., Isoo, N., Takasugi, N., Tsuruoka, M., Ui-Tei, K., Saigo, K., Morohashi, Y., Tomita, T., and Iwatsubo, T. (2005). Aph-1 contributes to the stabilization and trafficking of the gamma-secretase complex through mechanisms involving intermolecular and intramolecular interactions. J Biol Chem *280*, 12967-12975.

Nikolaev, A., McLaughlin, T., O'Leary, D.D., and Tessier-Lavigne, M. (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature *457*, 981-989.

Nimmrich, V., and Ebert, U. (2009). Is Alzheimer's disease a result of presynaptic failure? Synaptic dysfunctions induced by oligomeric beta-amyloid. Rev Neurosci *20*, 1-12.

Ninomiya, H., Roch, J.M., Sundsmo, M.P., Otero, D.A., and Saitoh, T. (1993). Amino acid sequence RERMS represents the active domain of amyloid beta/A4 protein precursor that promotes fibroblast growth. J Cell Biol *121*, 879-886.

Nishimura, I., Uetsuki, T., Kuwako, K., Hara, T., Kawakami, T., Aimoto, S., and Yoshikawa, K. (2002). Cell death induced by a caspase-cleaved transmembrane fragment of the Alzheimer amyloid precursor protein. Cell Death Differ *9*, 199-208.

Nuutinen, T., Suuronen, T., Kauppinen, A., and Salminen, A. (2009). Clusterin: a forgotten player in Alzheimer's disease. Brain Res Rev *61*, 89-104.

Oddo, S., Caccamo, A., Tran, L., Lambert, M.P., Glabe, C.G., Klein, W.L., and LaFerla, F.M. (2006). Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. A link between Abeta and tau pathology. J Biol Chem *281*, 1599-1604.

Ohkuma, S., and Poole, B. (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci U S A *75*, 3327-3331.

Olsson, A., Hoglund, K., Sjogren, M., Andreasen, N., Minthon, L., Lannfelt, L., Buerger, K., Moller, H.J., Hampel, H., Davidsson, P.*, et al.* (2003). Measurement of alpha- and beta-secretase cleaved amyloid precursor protein in cerebrospinal fluid from Alzheimer patients. Exp Neurol *183*, 74-80.

Otvos, L., Jr., Szendrei, G.I., Lee, V.M., and Mantsch, H.H. (1993). Human and rodent Alzheimer beta-amyloid peptides acquire distinct conformations in membrane-mimicking solvents. Eur J Biochem *211*, 249-257.

Pahlsson, P., and Spitalnik, S.L. (1996). The role of glycosylation in synthesis and secretion of beta-amyloid precursor protein by Chinese hamster ovary cells. Arch Biochem Biophys *331*, 177-186.

Pangalos, M.N., Efthimiopoulos, S., Shioi, J., and Robakis, N.K. (1995). The chondroitin sulfate attachment site of appican is formed by splicing out exon 15 of the amyloid precursor gene. J Biol Chem *270*, 10388-10391.

Parvathy, S., Hussain, I., Karran, E.H., Turner, A.J., and Hooper, N.M. (1999). Cleavage of Alzheimer's amyloid precursor protein by alpha-secretase occurs at the surface of neuronal cells. Biochemistry *38*, 9728-9734.

Pastorino, L., Ikin, A.F., Nairn, A.C., Pursnani, A., and Buxbaum, J.D. (2002). The carboxyl-terminus of BACE contains a sorting signal that regulates BACE trafficking but not the formation of total A(beta). Mol Cell Neurosci *19*, 175-185.

Patterson, D., Gardiner, K., Kao, F.T., Tanzi, R., Watkins, P., and Gusella, J.F. (1988). Mapping of the gene encoding the beta-amyloid precursor protein and its relationship to the Down syndrome region of chromosome 21. Proc Natl Acad Sci U S A *85*, 8266-8270.

Pellegrini, L., Passer, B., Tabaton, M., Ganjei, J., and D'Adamio, L. (1999). Alternative, non-secretase processing of Alzheimer's beta-amyloid precursor protein during apoptosis by caspase-6 and -8. J Biol Chem *274*, 21011-21016.

Perez, R.G., Zheng, H., Van der Ploeg, L.H., and Koo, E.H. (1997). The beta-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. J Neurosci *17*, 9407-9414.

Perl, D.P. (2010). Neuropathology of Alzheimer's disease. Mt Sinai J Med *77*, 32-42.

Pike, K.E., Savage, G., Villemagne, V.L., Ng, S., Moss, S.A., Maruff, P., Mathis, C.A., Klunk, W.E., Masters, C.L., and Rowe, C.C. (2007). Beta-amyloid imaging and memory in non-demented individuals: evidence for preclinical Alzheimer's disease. Brain *130*, 2837-2844.

Plassman, B.L., Langa, K.M., Fisher, G.G., Heeringa, S.G., Weir, D.R., Ofstedal, M.B., Burke, J.R., Hurd, M.D., Potter, G.G., Rodgers, W.L.*, et al.* (2007). Prevalence of dementia in the United States: the aging, demographics, and memory study. Neuroepidemiology *29*, 125-132.

Postina, R., Schroeder, A., Dewachter, I., Bohl, J., Schmitt, U., Kojro, E., Prinzen, C., Endres, K., Hiemke, C., Blessing, M.*, et al.* (2004). A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. J Clin Invest *113*, 1456-1464.

Price, D.L., Tanzi, R.E., Borchelt, D.R., and Sisodia, S.S. (1998). Alzheimer's disease: genetic studies and transgenic models. Annu Rev Genet *32*, 461-493.

Priola, S.A., Caughey, B., Wehrly, K., and Chesebro, B. (1995). A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. J Biol Chem *270*, 3299-3305.

Puertollano, R., and Bonifacino, J.S. (2004). Interactions of GGA3 with the ubiquitin sorting machinery. Nat Cell Biol *6*, 244-251.

Puzzo, D., Privitera, L., Leznik, E., Fa, M., Staniszewski, A., Palmeri, A., and Arancio, O. (2008). Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci *28*, 14537-14545.

Qing, H., Zhou, W., Christensen, M.A., Sun, X., Tong, Y., and Song, W. (2004). Degradation of BACE by the ubiquitin-proteasome pathway. FASEB J *18*, 1571-1573.

Querfurth, H.W., and LaFerla, F.M. (2010). Alzheimer's disease. N Engl J Med *362*, 329-344.

Rabinovici, G.D., Furst, A.J., O'Neil, J.P., Racine, C.A., Mormino, E.C., Baker, S.L., Chetty, S., Patel, P., Pagliaro, T.A., Klunk, W.E.*, et al.* (2007). 11C-PIB PET imaging in Alzheimer disease and frontotemporal lobar degeneration. Neurology *68*, 1205-1212.

Ramelot, T.A., and Nicholson, L.K. (2001). Phosphorylation-induced structural changes in the amyloid precursor protein cytoplasmic tail detected by NMR. J Mol Biol *307*, 871-884.

Ray, W.J., Yao, M., Mumm, J., Schroeter, E.H., Saftig, P., Wolfe, M., Selkoe, D.J., Kopan, R., and Goate, A.M. (1999). Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. J Biol Chem *274*, 36801-36807.

Reiman, E.M., Webster, J.A., Myers, A.J., Hardy, J., Dunckley, T., Zismann, V.L., Joshipura, K.D., Pearson, J.V., Hu-Lince, D., Huentelman, M.J.*, et al.* (2007). GAB2 alleles modify Alzheimer's risk in APOE epsilon4 carriers. Neuron *54*, 713-720.

Richards, S.J., Hodgman, C., and Sharpe, M. (1995). Reported sequence homology between Alzheimer amyloid770 and the MRC OX-2 antigen does not predict function. Brain Res Bull *38*, 305-306.

Richter, L., Munter, L.-M., Ness, J., Hildebrand, P.W., Dasari, M., Unterreitmeier, S., Bulic, B., Beyermann, M., Gust, R., Reif, B.*, et al.* (2010). Amyloid beta 42 peptide (A{beta}42)-lowering compounds directly bind to A{beta} and interfere with amyloid precursor protein (APP) transmembrane dimerization. Proceedings of the National Academy of Sciences of the United States of America.

Ridha, B.H., Barnes, J., Bartlett, J.W., Godbolt, A., Pepple, T., Rossor, M.N., and Fox, N.C. (2006). Tracking atrophy progression in familial Alzheimer's disease: a serial MRI study. Lancet Neurol *5*, 828-834.

Robakis, N.K., Wisniewski, H.M., Jenkins, E.C., Devine-Gage, E.A., Houck, G.E., Yao, X.L., Ramakrishna, N., Wolfe, G., Silverman, W.P., and Brown, W.T. (1987). Chromosome 21q21 sublocalisation of gene encoding beta-amyloid peptide in cerebral vessels and neuritic (senile) plaques of people with Alzheimer disease and Down syndrome. Lancet *1*, 384-385.

Roberts, G.W., Gentleman, S.M., Lynch, A., and Graham, D.I. (1991). beta A4 amyloid protein deposition in brain after head trauma. Lancet *338*, 1422-1423.

Roberts, G.W., Gentleman, S.M., Lynch, A., Murray, L., Landon, M., and Graham, D.I. (1994). Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. J Neurol Neurosurg Psychiatry *57*, 419-425.

Roch, J.M., Masliah, E., Roch-Levecq, A.C., Sundsmo, M.P., Otero, D.A., Veinbergs, I., and Saitoh, T. (1994). Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/A4 protein precursor. Proc Natl Acad Sci U S A *91*, 7450-7454.

Rogaeva, E., Meng, Y., Lee, J.H., Gu, Y., Kawarai, T., Zou, F., Katayama, T., Baldwin, C.T., Cheng, R., Hasegawa, H.*, et al.* (2007). The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet *39*, 168-177.

Rogers, J., Li, R., Mastroeni, D., Grover, A., Leonard, B., Ahern, G., Cao, P., Kolody, H., Vedders, L., Kolb, W.P.*, et al.* (2006). Peripheral clearance of amyloid beta peptide by complement C3-dependent adherence to erythrocytes. Neurobiol Aging *27*, 1733-1739.

Rohn, T., Ivins, K., Bahr, B., Cotman, C., and Cribbs, D. (2000). A monoclonal antibody to amyloid precursor protein induces neuronal apoptosis. J Neurochem *74*, 2331-2342.

Rosen, D.R., Martin-Morris, L., Luo, L.Q., and White, K. (1989). A Drosophila gene encoding a protein resembling the human beta-amyloid protein precursor. Proc Natl Acad Sci U S A *86*, 2478-2482.

Rossjohn, J., Cappai, R., Feil, S.C., Henry, A., McKinstry, W.J., Galatis, D., Hesse, L., Multhaup, G., Beyreuther, K., Masters, C.L.*, et al.* (1999). Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein. Nat Struct Biol *6*, 327-331.

Rowan, M.J., Klyubin, I., Wang, Q., Hu, N.W., and Anwyl, R. (2007). Synaptic memory mechanisms: Alzheimer's disease amyloid beta-peptide-induced dysfunction. Biochem Soc Trans *35*, 1219-1223.

Rowe, C.C., Ng, S., Ackermann, U., Gong, S.J., Pike, K., Savage, G., Cowie, T.F., Dickinson, K.L., Maruff, P., Darby, D.*, et al.* (2007). Imaging beta-amyloid burden in aging and dementia. Neurology *68*, 1718-1725.

Roychaudhuri, R., Yang, M., Hoshi, M.M., and Teplow, D.B. (2009). Amyloid beta-protein assembly and Alzheimer disease. J Biol Chem *284*, 4749-4753.

Russ, W.P., and Engelman, D.M. (2000). The GxxxG motif: a framework for transmembrane helix-helix association. J Mol Biol *296*, 911-919.

Saganich, M.J., Schroeder, B.E., Galvan, V., Bredesen, D.E., Koo, E.H., and Heinemann, S.F. (2006). Deficits in synaptic transmission and learning in amyloid precursor protein (APP) transgenic mice require C-terminal cleavage of APP. J Neurosci *26*, 13428-13436.

Saitoh, T., Sundsmo, M., Roch, J.M., Kimura, N., Cole, G., Schubert, D., Oltersdorf, T., and Schenk, D.B. (1989). Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts. Cell *58*, 615-622.

Sakono, M., and Zako, T. (2010). Amyloid oligomers: formation and toxicity of Abeta oligomers. FEBS J *277*, 1348-1358.

Sambamurti, K., Shioi, J., Anderson, J.P., Pappolla, M.A., and Robakis, N.K. (1992). Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. J Neurosci Res *33*, 319-329.

Sandbrink, R., Masters, C.L., and Beyreuther, K. (1994). Similar alternative splicing of a non-homologous domain in beta A4-amyloid protein precursor-like proteins. J Biol Chem *269*, 14227-14234.

Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M.M., Teplow, D.B., and Haass, C. (2001). Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. EMBO Rep *2*, 835-841.

Sato, T., Kienlen-Campard, P., Ahmed, M., Liu, W., Li, H., Elliott, J.I., Aimoto, S., Constantinescu, S.N., Octave, J.N., and Smith, S.O. (2006). Inhibitors of amyloid toxicity based on beta-sheet packing of Abeta40 and Abeta42. Biochemistry *45*, 5503-5516.

Satterlee, J.S., Barbee, S., Jin, P., Krichevsky, A., Salama, S., Schratt, G., and Wu, D.Y. (2007). Noncoding RNAs in the brain. J Neurosci *27*, 11856-11859.

Scheff, S.W., and Price, D.A. (1993). Synapse loss in the temporal lobe in Alzheimer's disease. Ann Neurol *33*, 190-199.

Scheff, S.W., and Price, D.A. (1998). Synaptic density in the inner molecular layer of the hippocampal dentate gyrus in Alzheimer disease. J Neuropathol Exp Neurol *57*, 1146-1153.

Scheff, S.W., and Price, D.A. (2006). Alzheimer's disease-related alterations in synaptic density: neocortex and hippocampus. J Alzheimers Dis *9*, 101-115.

Scheff, S.W., Price, D.A., and Sparks, D.L. (2001). Quantitative assessment of possible age-related change in synaptic numbers in the human frontal cortex. Neurobiol Aging *22*, 355-365.

Scheltens, P., and Korf, E.S. (2000). Contribution of neuroimaging in the diagnosis of Alzheimer's disease and other dementias. Curr Opin Neurol *13*, 391-396.

Scheuermann, S., Hambsch, B., Hesse, L., Stumm, J., Schmidt, C., Beher, D., Bayer, T.A., Beyreuther, K., and Multhaup, G. (2001). Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease. The Journal of biological chemistry *276*, 33923-33929.

Schilling, S., Zeitschel, U., Hoffmann, T., Heiser, U., Francke, M., Kehlen, A., Holzer, M., Hutter-Paier, B., Prokesch, M., Windisch, M.*, et al.* (2008). Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology. Nat Med *14*, 1106-1111.

Schjeide, B.M., Schnack, C., Lambert, J.C., Lill, C.M., Kirchheiner, J., Hayret- tin, T., Otto, M., Tanzi, R.E., L., H., and Amouyel, P. (in press). The role of CLU, CR1, and PICALM on Alzheimer's disease risk and CSF biomarker levels.

Schott, J.M., Fox, N.C., Frost, C., Scahill, R.I., Janssen, J.C., Chan, D., Jenkins, R., and Rossor, M.N. (2003). Assessing the onset of structural change in familial Alzheimer's disease. Ann Neurol *53*, 181-188.

Schubert, D., and Behl, C. (1993). The expression of amyloid beta protein precursor protects nerve cells from beta-amyloid and glutamate toxicity and alters their interaction with the extracellular matrix. Brain Res *629*, 275-282.

Schubert, D., Jin, L.W., Saitoh, T., and Cole, G. (1989). The regulation of amyloid beta protein precursor secretion and its modulatory role in cell adhesion. Neuron *3*, 689-694.

Selkoe, D.J. (2001). Clearing the brain's amyloid cobwebs. Neuron *32*, 177-180.

Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. Science *298*, 789-791.

Sennvik, K., Fastbom, J., Blomberg, M., Wahlund, L.O., Winblad, B., and Benedikz, E. (2000). Levels of alpha- and beta-secretase cleaved amyloid precursor protein in the cerebrospinal fluid of Alzheimer's disease patients. Neurosci Lett *278*, 169-172.

Shah, S., Lee, S.F., Tabuchi, K., Hao, Y.H., Yu, C., LaPlant, Q., Ball, H., Dann, C.E., 3rd, Sudhof, T., and Yu, G. (2005). Nicastrin functions as a gamma-secretase-substrate receptor. Cell *122*, 435-447.

Shaked, G.M., Kummer, M.P., Lu, D.C., Galvan, V., Bredesen, D.E., and Koo, E.H. (2006). Abeta induces cell death by direct interaction with its cognate extracellular domain on APP (APP 597-624). FASEB J *20*, 1254-1256.

Shankar, G.M., Bloodgood, B.L., Townsend, M., Walsh, D.M., Selkoe, D.J., and Sabatini, B.L. (2007). Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci *27*, 2866-2875.

Shankar, G.M., Li, S., Mehta, T.H., Garcia-Munoz, A., Shepardson, N.E., Smith, I., Brett, F.M., Farrell, M.A., Rowan, M.J., Lemere, C.A.*, et al.* (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med *14*, 837-842.

Sharon, D., Chen, R., and Snyder, M. (2010). Systems biology approaches to disease marker discovery. Dis Markers *28*, 209-224.

Shiba, T., Kametaka, S., Kawasaki, M., Shibata, M., Waguri, S., Uchiyama, Y., and Wakatsuki, S. (2004). Insights into the phosphoregulation of beta-secretase sorting signal by the VHS domain of GGA1. Traffic *5*, 437-448.

Shirotani, K., Edbauer, D., Kostka, M., Steiner, H., and Haass, C. (2004). Immature nicastrin stabilizes APH-1 independent of PEN-2 and presenilin: identification of nicastrin mutants that selectively interact with APH-1. J Neurochem *89*, 1520-1527.

Shrestha, B.R., Vitolo, O.V., Joshi, P., Lordkipanidze, T., Shelanski, M., and Dunaevsky, A. (2006). Amyloid beta peptide adversely affects spine number and motility in hippocampal neurons. Mol Cell Neurosci *33*, 274-282.

Siemes, C., Quast, T., Kummer, C., Wehner, S., Kirfel, G., Muller, U., and Herzog, V. (2006). Keratinocytes from APP/APLP2-deficient mice are impaired in proliferation, adhesion and migration in vitro. Exp Cell Res *312*, 1939-1949.

Simons, M., de Strooper, B., Multhaup, G., Tienari, P.J., Dotti, C.G., and Beyreuther, K. (1996). Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. J Neurosci *16*, 899-908.

Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J.*, et al.* (1999). Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature *402*, 537-540.

Sisodia, S.S. (1992). Beta-amyloid precursor protein cleavage by a membrane-bound protease. Proc Natl Acad Sci U S A *89*, 6075-6079.

Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A., and Price, D.L. (1990). Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing. Science *248*, 492-495.

Slunt, H.H., Thinakaran, G., Von Koch, C., Lo, A.C., Tanzi, R.E., and Sisodia, S.S. (1994). Expression of a ubiquitous, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). J Biol Chem *269*, 2637-2644.

Small, D.H., Nurcombe, V., Reed, G., Clarris, H., Moir, R., Beyreuther, K., and Masters, C.L. (1994). A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. J Neurosci *14*, 2117-2127.

Smith, M.A. (1998). Alzheimer disease. Int Rev Neurobiol *42*, 1-54.

Smith, S.O., Song, D., Shekar, S., Groesbeek, M., Ziliox, M., and Aimoto, S. (2001). Structure of the transmembrane dimer interface of glycophorin A in membrane bilayers. Biochemistry *40*, 6553-6558.

Smith-Swintosky, V.L., Pettigrew, L.C., Craddock, S.D., Culwell, A.R., Rydel, R.E., and Mattson, M.P. (1994). Secreted forms of beta-amyloid precursor protein protect against ischemic brain injury. J Neurochem *63*, 781-784.

Snow, A.D., and Kisilevsky, R. (1985). Temporal relationship between glycosaminoglycan accumulation and amyloid deposition during experimental amyloidosis. A histochemical study. Lab Invest *53*, 37-44.

Snow, A.D., Mar, H., Nochlin, D., Kimata, K., Kato, M., Suzuki, S., Hassell, J., and Wight, T.N. (1988). The presence of heparan sulfate proteoglycans in the neuritic plaques and congophilic angiopathy in Alzheimer's disease. Am J Pathol *133*, 456-463.

Snow, A.D., Mar, H., Nochlin, D., Sekiguchi, R.T., Kimata, K., Koike, Y., and Wight, T.N. (1990). Early accumulation of heparan sulfate in neurons and in the beta-amyloid

protein-containing lesions of Alzheimer's disease and Down's syndrome. Am J Pathol *137*, 1253-1270.

Snow, A.D., Sekiguchi, R., Nochlin, D., Fraser, P., Kimata, K., Mizutani, A., Arai, M., Schreier, W.A., and Morgan, D.G. (1994). An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain. Neuron *12*, 219-234.

Soba, P., Eggert, S., Wagner, K., Zentgraf, H., Siehl, K., Kreger, S., Löwer, A., Langer, A., Merdes, G., Paro, R.*, et al.* (2005). Homo- and heterodimerization of APP family members promotes intercellular adhesion. EMBO J *24*, 3624-3634.

Sodhi, C.P., Rampalli, S., Perez, R.G., Koo, E.H., Quinn, B., and Gottardi-Littell, N.R. (2004). The endocytotic pathway is required for increased A beta 42 secretion during apoptosis. Brain Res Mol Brain Res *128*, 201-211.

Sondag, C.M., and Combs, C.K. (2004). Amyloid precursor protein mediates proinflammatory activation of monocytic lineage cells. J Biol Chem *279*, 14456-14463.

Sondag, C.M., and Combs, C.K. (2006). Amyloid precursor protein cross-linking stimulates beta amyloid production and pro-inflammatory cytokine release in monocytic lineage cells. J Neurochem *97*, 449-461.

Steinbach, J.P., Muller, U., Leist, M., Li, Z.W., Nicotera, P., and Aguzzi, A. (1998). Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice. Cell Death Differ *5*, 858-866.

Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M.G., Lincoln, S., Hardy, J., Yu, X., Picciano, M., Fechteler, K.*, et al.* (1999). A loss of function mutation of presenilin-2 interferes with amyloid beta-peptide production and notch signaling. J Biol Chem *274*, 28669-28673.

Storey, E., Katz, M., Brickman, Y., Beyreuther, K., and Masters, C.L. (1999). Amyloid precursor protein of Alzheimer's disease: evidence for a stable, full-length, trans-membrane pool in primary neuronal cultures. Eur J Neurosci *11*, 1779-1788.

Storey, E., Spurck, T., Pickett-Heaps, J., Beyreuther, K., and Masters, C.L. (1996). The amyloid precursor protein of Alzheimer's disease is found on the surface of static but not activity motile portions of neurites. Brain Res *735*, 59-66.

Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S., and Roses, A.D. (1993). Apolipoprotein E: high-avidity binding to

beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A *90*, 1977-1981.

Struhl, G., and Adachi, A. (2000). Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol Cell *6*, 625-636.

Suzuki, N., Cheung, T.T., Cai, X.D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T.E., and Younkin, S.G. (1994). An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science *264*, 1336-1340.

Tabaton, M., Zhu, X., Perry, G., Smith, M.A., and Giliberto, L. (2010). Signaling effect of amyloid-beta(42) on the processing of AbetaPP. Exp Neurol *221*, 18-25.

Takahashi, H., Mizui, T., and Shirao, T. (2006). Down-regulation of drebrin A expression suppresses synaptic targeting of NMDA receptors in developing hippocampal neurones. J Neurochem *97 Suppl 1*, 110-115.

Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003). The role of presenilin cofactors in the gamma-secretase complex. Nature *422*, 438-441.

Tamagno, E., Bardini, P., Obbili, A., Vitali, A., Borghi, R., Zaccheo, D., Pronzato, M.A., Danni, O., Smith, M.A., Perry, G.*, et al.* (2002). Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol Dis *10*, 279-288.

Tampellini, D., Magrané, J., Takahashi, R.H., Li, F., Lin, M.T., Almeida, C.G., and Gouras, G.K. (2007). Internalized antibodies to the Abeta domain of APP reduce neuronal Abeta and protect against synaptic alterations. The Journal of biological chemistry *282*, 18895-18906.

Tang, B.L. (2009). Neuronal protein trafficking associated with Alzheimer disease: from APP and BACE1 to glutamate receptors. Cell Adh Migr *3*, 118-128.

Tanzi, R.E., and Bertram, L. (2005). Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell *120*, 545-555.

Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A., St George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M., and Neve, R.L. (1987). Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science *235*, 880-884.

Taru, H., Yoshikawa, K., and Suzuki, T. (2004). Suppression of the caspase cleavage of beta-amyloid precursor protein by its cytoplasmic phosphorylation. FEBS Lett *567*, 248-252.

Taylor, C.J., Ireland, D.R., Ballagh, I., Bourne, K., Marechal, N.M., Turner, P.R., Bilkey, D.K., Tate, W.P., and Abraham, W.C. (2008). Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. Neurobiol Dis *31*, 250-260.

Tebar, F., Bohlander, S.K., and Sorkin, A. (1999). Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. Mol Biol Cell *10*, 2687-2702.

Terry, R.D. (1986). Interrelations among the lesions of normal and abnormal aging of the brain. Prog Brain Res *70*, 41-48.

Terry, R.D., Gonatas, N.K., and Weiss, M. (1964). Ultrastructural Studies in Alzheimer's Presenile Dementia. Am J Pathol *44*, 269-297.

Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., Hansen, L.A., and Katzman, R. (1991). Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol *30*, 572-580.

Tesco, G., Koh, Y.H., Kang, E.L., Cameron, A.N., Das, S., Sena-Esteves, M., Hiltunen, M., Yang, S.-H., Zhong, Z., Shen, Y.*, et al.* (2007). Depletion of GGA3 Stabilizes BACE and Enhances beta-Secretase Activity. Neuron *54*, 721-737.

Tesco, G., Koh, Y.H., and Tanzi, R.E. (2003). Caspase activation increases beta-amyloid generation independently of caspase cleavage of the beta-amyloid precursor protein (APP). J Biol Chem *278*, 46074-46080.

Thambisetty, M., Simmons, A., Velayudhan, L., Hye, A., Campbell, J., Zhang, Y., Wahlund, L.O., Westman, E., Kinsey, A., Guntert, A.*, et al.* (2010). Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. Arch Gen Psychiatry *67*, 739-748.

Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M.*, et al.* (1996a). Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron *17*, 181-190.

Thinakaran, G., and Koo, E. (2008). APP trafficking, processing and function. The Journal of biological chemistry.

Thinakaran, G., Teplow, D.B., Siman, R., Greenberg, B., and Sisodia, S.S. (1996b). Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a)

cells. Evidence that cleavage at the "beta-secretase" site occurs in the golgi apparatus. J Biol Chem *271*, 9390-9397.

Townsend, Mehta, and Selkoe (2007). Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. J Biol Chem.

Troy, C.M., Rabacchi, S.A., Friedman, W.J., Frappier, T.F., Brown, K., and Shelanski, M.L. (2000). Caspase-2 mediates neuronal cell death induced by beta-amyloid. J Neurosci *20*, 1386-1392.

Tseng, B.P., Green, K.N., Chan, J.L., Blurton-Jones, M., and LaFerla, F.M. (2008). Abeta inhibits the proteasome and enhances amyloid and tau accumulation. Neurobiol Aging *29*, 1607-1618.

Tu, S., McStay, G.P., Boucher, L.M., Mak, T., Beere, H.M., and Green, D.R. (2006). In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. Nat Cell Biol *8*, 72-77.

Turk, E., Teplow, D.B., Hood, L.E., and Prusiner, S.B. (1988). Purification and properties of the cellular and scrapie hamster prion proteins. Eur J Biochem *176*, 21-30.

Turner, P.R., O'Connor, K., Tate, W.P., and Abraham, W.C. (2003). Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol *70*, 1-32.

Tyler, S.J., Dawbarn, D., Wilcock, G.K., and Allen, S.J. (2002). alpha- and beta-secretase: profound changes in Alzheimer's disease. Biochem Biophys Res Commun *299*, 373-376.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-212.

Unger, J.W., Lapham, L.W., McNeill, T.H., Eskin, T.A., and Hamill, R.W. (1991). The amygdala in Alzheimer's disease: neuropathology and Alz 50 immunoreactivity. Neurobiol Aging *12*, 389-399.

Valincius, G., Heinrich, F., Budvytyte, R., Vanderah, D.J., McGillivray, D.J., Sokolov, Y., Hall, J.E., and Losche, M. (2008). Soluble amyloid beta-oligomers affect dielectric membrane properties by bilayer insertion and domain formation: implications for cell toxicity. Biophys J *95*, 4845-4861.

van Horssen, J., Wesseling, P., van den Heuvel, L.P., de Waal, R.M., and Verbeek, M.M. (2003). Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. Lancet Neurol *2*, 482-492.

Vance, J.E., and Hayashi, H. (2010). Formation and function of apolipoprotein E-containing lipoproteins in the nervous system. Biochim Biophys Acta *1801*, 806-818.

Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R.*, et al.* (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science *286*, 735-741.

Velliquette, R.A., O'Connor, T., and Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. J Neurosci *25*, 10874-10883.

Vetrivel, K.S., Cheng, H., Lin, W., Sakurai, T., Li, T., Nukina, N., Wong, P.C., Xu, H., and Thinakaran, G. (2004). Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem *279*, 44945-44954.

Vetrivel, K.S., and Thinakaran, G. (2010). Membrane rafts in Alzheimer's disease beta-amyloid production. Biochim Biophys Acta *1801*, 860-867.

Virchow, R. (1853). Virchow's Arch *6*, 135, 268, 416.

Vitolo, O.V., Sant'Angelo, A., Costanzo, V., Battaglia, F., Arancio, O., and Shelanski, M. (2002). Amyloid beta -peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling. Proc Natl Acad Sci USA *99*, 13217-13221.

von Arnim, C.A., Tangredi, M.M., Peltan, I.D., Lee, B.M., Irizarry, M.C., Kinoshita, A., and Hyman, B.T. (2004). Demonstration of BACE (beta-secretase) phosphorylation and its interaction with GGA1 in cells by fluorescence-lifetime imaging microscopy. J Cell Sci *117*, 5437-5445.

von der Kammer, H., Loffler, C., Hanes, J., Klaudiny, J., Scheit, K.H., and Hansmann, I. (1994). The gene for the amyloid precursor-like protein APLP2 is assigned to human chromosome 11q23-q25. Genomics *20*, 308-311.

von Koch, C.S., Zheng, H., Chen, H., Trumbauer, M., Thinakaran, G., van der Ploeg, L.H., Price, D.L., and Sisodia, S.S. (1997). Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. Neurobiol Aging *18*, 661-669.

Wahle, T., Prager, K., Raffler, N., Haass, C., Famulok, M., and Walter, J. (2005). GGA proteins regulate retrograde transport of BACE1 from endosomes to the trans-Golgi network. Mol Cell Neurosci *29*, 453-461.

Wahle, T., Thal, D.R., Sastre, M., Rentmeister, A., Bogdanovic, N., Famulok, M., Heneka, M.T., and Walter, J. (2006). GGA1 is expressed in the human brain and affects the generation of amyloid beta-peptide. J Neurosci *26*, 12838-12846.

Walsh, D.M., Minogue, A.M., Sala Frigerio, C., Fadeeva, J.V., Wasco, W., and Selkoe, D.J. (2007). The APP family of proteins: similarities and differences. Biochem Soc Trans *35*, 416-420.

Walsh, D.M., Tseng, B.P., Rydel, R.E., Podlisny, M.B., and Selkoe, D.J. (2000). The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry *39*, 10831-10839.

Walter, J., Fluhrer, R., Hartung, B., Willem, M., Kaether, C., Capell, A., Lammich, S., Multhaup, G., and Haass, C. (2001). Phosphorylation regulates intracellular trafficking of beta-secretase. J Biol Chem *276*, 14634-14641.

Walton, J.R., and Wang, M.X. (2009). APP expression, distribution and accumulation are altered by aluminum in a rodent model for Alzheimer's disease. J Inorg Biochem *103*, 1548-1554.

Wang, H.W., Pasternak, J.F., Kuo, H., Ristic, H., Lambert, M.P., Chromy, B., Viola, K.L., Klein, W.L., Stine, W.B., Krafft, G.A.*, et al.* (2002). Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. Brain Res *924*, 133-140.

Wang, Y., and Ha, Y. (2004). The X-ray structure of an antiparallel dimer of the human amyloid precursor protein E2 domain. Mol Cell *15*, 343-353.

Wang, Z.F., Li, H.L., Li, X.C., Zhang, Q., Tian, Q., Wang, Q., Xu, H., and Wang, J.Z. (2006). Effects of endogenous beta-amyloid overproduction on tau phosphorylation in cell culture. J Neurochem *98*, 1167-1175.

Wasco, W., Brook, J.D., and Tanzi, R.E. (1993a). The amyloid precursor-like protein (APLP) gene maps to the long arm of human chromosome 19. Genomics *15*, 237-239.

Wasco, W., Bupp, K., Magendantz, M., Gusella, J.F., Tanzi, R.E., and Solomon, F. (1992). Identification of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid beta protein precursor. Proc Natl Acad Sci U S A *89*, 10758-10762.

Wasco, W., Gurubhagavatula, S., Paradis, M.D., Romano, D.M., Sisodia, S.S., Hyman, B.T., Neve, R.L., and Tanzi, R.E. (1993b). Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor. Nat Genet *5*, 95-100.

Weggen, S., Eriksen, J.L., Das, P., Sagi, S.A., Wang, R., Pietrzik, C.U., Findlay, K.A., Smith, T.E., Murphy, M.P., Bulter, T.*, et al.* (2001). A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature *414*, 212-216.

Weidemann, A., Eggert, S., Reinhard, F.B., Vogel, M., Paliga, K., Baier, G., Masters, C.L., Beyreuther, K., and Evin, G. (2002). A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. Biochemistry *41*, 2825-2835.

Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L., and Beyreuther, K. (1989). Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. Cell *57*, 115-126.

Wen, Y., Onyewuchi, O., Yang, S., Liu, R., and Simpkins, J.W. (2004). Increased beta-secretase activity and expression in rats following transient cerebral ischemia. Brain Res *1009*, 1-8.

Wen, Y., Yu, W.H., Maloney, B., Bailey, J., Ma, J., Mari\'e, I., Maurin, T., Wang, L., Figueroa, H., Herman, M.*, et al.* (2008). Transcriptional Regulation of beta-Secretase by p25/cdk5 Leads to Enhanced Amyloidogenic Processing. Neuron *57*, 680-690.

White, A.R., Reyes, R., Mercer, J.F., Camakaris, J., Zheng, H., Bush, A.I., Multhaup, G., Beyreuther, K., Masters, C.L., and Cappai, R. (1999). Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. Brain Res *842*, 439-444.

White, A.R., Zheng, H., Galatis, D., Maher, F., Hesse, L., Multhaup, G., Beyreuther, K., Masters, C.L., and Cappai, R. (1998). Survival of cultured neurons from amyloid precursor protein knock-out mice against Alzheimer's amyloid-beta toxicity and oxidative stress. J Neurosci *18*, 6207-6217.

Wilcock, G.K., and Esiri, M.M. (1982). Plaques, tangles and dementia. A quantitative study. J Neurol Sci *56*, 343-356.

Williamson, T.G., Mok, S.S., Henry, A., Cappai, R., Lander, A.D., Nurcombe, V., Beyreuther, K., Masters, C.L., and Small, D.H. (1996). Secreted glypican binds to the amyloid precursor protein of Alzheimer's disease (APP) and inhibits APP-induced neurite outgrowth. J Biol Chem *271*, 31215-31221.

Williamson, T.G., Nurcombe, V., Beyreuther, K., Masters, C.L., and Small, D.H. (1995). Affinity purification of proteoglycans that bind to the amyloid protein precursor of Alzheimer's disease. J Neurochem *65*, 2201-2208.

Wisniewski, H.M., Bancher, C., Barcikowska, M., Wen, G.Y., and Currie, J. (1989). Spectrum of morphological appearance of amyloid deposits in Alzheimer's disease. Acta Neuropathol *78*, 337-347.

Wisniewski, H.M., Narang, H.K., and Terry, R.D. (1976). Neurofibrillary tangles of paired helical filaments. J Neurol Sci *27*, 173-181.

Wisniewski, H.M., Vorbrodt, A.W., Moretz, R.C., Lossinsky, A.S., and Grundke-Iqbal, I. (1982). Pathogenesis of neuritic (senile) and amyloid plaque formation. Exp Brain Res *Suppl 5*, 3-9.

Wisniewski, K., Jervis, G.A., Moretz, R.C., and Wisniewski, H.M. (1979). Alzheimer neurofibrillary tangles in diseases other than senile and presenile dementia. Ann Neurol *5*, 288-294.

Wolfe, M.S. (2008). Inhibition and modulation of gamma-secretase for Alzheimer's disease. Neurotherapeutics *5*, 391-398.

Wong, C.W., Quaranta, V., and Glenner, G.G. (1985). Neuritic plaques and cerebrovascular amyloid in Alzheimer disease are antigenically related. Proc Natl Acad Sci U S A *82*, 8729-8732.

Wood, J.G., Mirra, S.S., Pollock, N.J., and Binder, L.I. (1986). Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau (tau). Proc Natl Acad Sci U S A *83*, 4040-4043.

Wyss-Coray, T., Yan, F., Lin, A.H., Lambris, J.D., Alexander, J.J., Quigg, R.J., and Masliah, E. (2002). Prominent neurodegeneration and increased plaque formation in complement-inhibited Alzheimer's mice. Proc Natl Acad Sci U S A *99*, 10837-10842.

Xu, F., Davis, J., Miao, J., Previti, M.L., Romanov, G., Ziegler, K., and Van Nostrand, W.E. (2005). Protease nexin-2/amyloid beta-protein precursor limits cerebral thrombosis. Proc Natl Acad Sci U S A *102*, 18135-18140.

Xu, H., Sweeney, D., Wang, R., Thinakaran, G., Lo, A.C., Sisodia, S.S., Greengard, P., and Gandy, S. (1997). Generation of Alzheimer beta-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. Proc Natl Acad Sci U S A *94*, 3748-3752.

Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., and Nakazato, Y. (1989). Diffuse type of senile plaques in the cerebellum of Alzheimer-type dementia demonstrated by beta protein immunostain. Acta Neuropathol *77*, 314-319.

Yamazaki, T., Koo, E.H., and Selkoe, D.J. (1997). Cell surface amyloid beta-protein precursor colocalizes with beta 1 integrins at substrate contact sites in neural cells. J Neurosci *17*, 1004-1010.

Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashier, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E.*, et al.* (1999). Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature *402*, 533-537.

Yang, L.B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X.L., Beach, T., Sue, L., Wong, P., Price, D.*, et al.* (2003). Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat Med *9*, 3-4.

Yao, P.J., Petralia, R.S., Bushlin, I., Wang, Y., and Furukawa, K. (2005). Synaptic distribution of the endocytic accessory proteins AP180 and CALM. J Comp Neurol *481*, 58-69.

Yasojima, K., Akiyama, H., McGeer, E.G., and McGeer, P.L. (2001). Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of beta-amyloid peptide. Neurosci Lett *297*, 97-100.

Yoshikai, S., Sasaki, H., Doh-ura, K., Furuya, H., and Sakaki, Y. (1991). Genomic organization of the human-amyloid beta-protein precursor gene. Gene *102*, 291-292.

Young-Pearse, T.L., Bai, J., Chang, R., Zheng, J.B., LoTurco, J.J., and Selkoe, D.J. (2007). A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J Neurosci *27*, 14459-14469.

Young-Pearse, T.L., Chen, A.C., Chang, R., Marquez, C., and Selkoe, D.J. (2008). Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1. Neural Dev *3*, 15.

Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D.M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Duthie, M.*, et al.* (1998). The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin. J Biol Chem *273*, 16470-16475.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T.*, et al.* (2000). Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature *407*, 48-54.

Zhang, X., Zhou, K., Wang, R., Cui, J., Lipton, S.A., Liao, F.F., Xu, H., and Zhang, Y.W. (2007). Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia

increases BACE1 expression and beta-amyloid generation. J Biol Chem *282*, 10873-10880.

Zhang, Y., Hong, Y., Bounhar, Y., Blacker, M., Roucou, X., Tounekti, O., Vereker, E., Bowers, W.J., Federoff, H.J., Goodyer, C.G.*, et al.* (2003). p75 neurotrophin receptor protects primary cultures of human neurons against extracellular amyloid beta peptide cytotoxicity. J Neurosci *23*, 7385-7394.

Zhang, Y., McLaughlin, R., Goodyer, C., and LeBlanc, A. (2002). Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. J Cell Biol *156*, 519-529.

Zhang, Y.W., and Xu, H. (2007). Molecular and cellular mechanisms for Alzheimer's disease: understanding APP metabolism. Curr Mol Med *7*, 687-696.

Zhao, G., Mao, G., Tan, J., Dong, Y., Cui, M.Z., Kim, S.H., and Xu, X. (2004). Identification of a new presenilin-dependent zeta-cleavage site within the transmembrane domain of amyloid precursor protein. J Biol Chem *279*, 50647-50650.

Zhao, J., Fu, Y., Yasvoina, M., Shao, P., Hitt, B., O'Connor, T., Logan, S., Maus, E., Citron, M., Berry, R.*, et al.* (2007). Beta-site amyloid precursor protein cleaving enzyme 1 levels become elevated in neurons around amyloid plaques: implications for Alzheimer's disease pathogenesis. J Neurosci *27*, 3639-3649.

Zhao, M., Su, J., Head, E., and Cotman, C.W. (2003). Accumulation of caspase cleaved amyloid precursor protein represents an early neurodegenerative event in aging and in Alzheimer's disease. Neurobiol Dis *14*, 391-403.

Zhao, W.-Q.Q., De Felice, F.G., Fernandez, S., Chen, H., Lambert, M.P., Quon, M.J., Krafft, G.A., and Klein, W.L. (2008). Amyloid beta oligomers induce impairment of neuronal insulin receptors. FASEB J *22*, 246-260.

Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W.*, et al.* (1995). beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell *81*, 525-531.

Zlokovic, B.V. (1996). Cerebrovascular transport of Alzheimer's amyloid beta and apolipoproteins J and E: possible anti-amyloidogenic role of the blood-brain barrier. Life Sci *59*, 1483-1497.