Aβ oligomer-mediated calcium influx via the N-methyl-D-aspartate receptor connects excitotoxic signaling to the cell cycle re-entry pathway of neuron death in Alzheimer's disease

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A Dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

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University of Virginia
March 2018

Abstract

Alzheimer's disease (AD) is a devastating neurological disorder characterized by progressive memory loss and cognitive decline. Histopathologically, AD is characterized by two types of protein aggregates in brain, amyloid-β (Aβ) plaques and tau-containing neurofibrillary tangles, as well as by massive synaptic loss and neuron death. Aβ peptides act upstream of tau in AD pathogenesis, and have been demonstrated to interact with multiple proteins at the cell surface. In particular, Aβ oligomers (AβOs) have been shown to interact with and cause calcium influx thorough the N-methyl-D-aspartate receptor. Neurons in adult brain are normally in a permanently post-mitotic state, but in AD they exhibit ectopic cell cycle re-entry (CCR), which leads to their eventual death.

The data presented in the following dissertation demonstrates that Aβ-mediated calcium influx through NMDAR is necessary for the induction of CCR in neurons, connecting the calcium dysfunction in AD to the CCR pathway of neuron death. Chelating total intracellular calcium in neurons with BAPTA-AM treatment inhibits CCR, indicating CCR is calcium dependent. When primary neurons are treated with the pharmacological inhibitor MK-801, specifically blocking calcium influx through the NMDAR channel pore, CCR is also inhibited, implying a critical role for NMDAR calcium influx in CCR pathogenesis. Additionally, AβO treatment causes early activation of CaMKII, a calcium-dependent protein kinase that must phosphorylate tau at Ser416 for CCR to occur. Blocking NMDAR-mediated calcium influx by MK-801 treatment blocks this CaMKII activation, putting NMDAR upstream of both CaMKII activation and subsequent phosphorylation of tau. Treatment of primary neurons with memantine, an FDA approved drug for the treatment of moderate to severe AD that blocks NMDAR

calcium influx, also inhibits CCR. Furthermore, treatment of the AD mouse model Tg2576 with memantine before the onset of AD pathology inhibits CCR in these mice, suggesting that memantine has a previously undemonstrated prophylactic role in AD treatment.

Together, these results indicate that excess calcium influx via the NMDA receptor is an essential early step in AβO-induced neuronal CCR. Furthermore, the collective data imply that this specific type of calcium dysregulation triggers the two cell biological responses that together account for the behavioral deficits in AD: excitotoxicity at the synapse, and neuron death (via CCR).

Dedication

I would like to first thank my advisor, George Bloom, for the advice, reassurance, and guidance he has provided to me over the course of my PhD. In George's lab, I had the opportunity to explore my own scientific ideas, and learn numerous techniques that I will be able to apply to my future scientific pursuits. George has always provided me with encouragement and direction that was invaluable to my scientific career, and I'm grateful for all of the work he has put into my training. Additionally, I am thankful for everyone I worked with in the Bloom lab. Over the years, these people have become my good friends, and were always willing to help me out. I am incredibly grateful to have had George and everyone in the lab supporting me throughout my graduate career. I thank Sophie Choi for all of the work she put into helping me with experiments in this thesis.

I would also like to thank my thesis committee, Dr. Iggy Provencio, Dr. Chris Deppmann, Dr. Ali Güler, and Dr. Jim Mandell for their constant support, and for all of their contributions and ideas that went into completing my thesis. I would especially like to thank Iggy for his support as my first reader and all of the advice he has provided me over the years. I am thankful to have found my way into the Biology department and could not have asked for a more supportive and welcoming group of scientists and staff.

Next, I would like to thank my parents for supporting me in all of my endeavors that led me to graduate school. Throughout high school and college and graduate school, they always encouraged me to do what I was passionate about and gave me the support and guidance I needed to make my dreams come true. I would not have made it to or through graduate school without their support, and I am immensely grateful for

everything they have given me. I would also like to thank my brother Jeff, for always being there for me, for giving me perspective on the world outside of biology, and for being one of my best friends. I would also like to thank all of my friends, in and out of graduate school, who were the best support system I could have asked for.

Lastly, I would like to thank my boyfriend, Chuck Schmidt, for his constant encouragement in everything, but especially in the work that went into my experiments and writing my thesis. His constant support got me to where I am today. He also made me dinner every night that I was writing my thesis, and made sure I had everything I needed while I was writing, for which I am incredibly grateful.

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List of Abbreviations:

2-APB	2-aminoethoxydiphenyl borate
Aβ	Amyloid-β
AβO	Amyloid-β oligomer
AD	Alzheimer's disease
ADDL	Amyloid-beta derived diffusible ligand
AICD	APP intracellular domain
AMPA	α-amino-methyl-propanoic acid
APOE	Apolipoprotein-E
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CaMKII	Calcium-calmodulin dependent kinase II
CCR	Cell cycle re-entry
CDK	Cyclin-dependent kinase
CICR	Calcium-induced calcium release
CNS	Central nervous system
CSF	Cerebral spinal fluid
CTF	C-terminal fragment
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution

HFIP	1,1,1,3,3,3-hexafluoroisopropanol
IHC	Immunohistochemistry
KD	Knock down
KO	Knock out
LTD	Long-term depression
LTP	Long-term potentiation
MAP	Microtubule-associated protein
MCI	Mild cognitive impairment
MTBD	Microtubule binding domain
NGF	Nerve growth factor
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PBST	PBS + 0.1% Tween-20
PCNA	Proliferating cell nuclear antigen
PHF	Paired helical filament
PiB	Pittsburgh compound B
PKA	Cyclic AMP-dependent protein kinase A
PNS	Peripheral nervous system
PS-1	Presenilin-1
PSD95	Postsynaptic density protein 95

Rb	Retinoblastoma protein
ROS	Reactive oxygen species
sAPP-α	Soluble ectodomain α
sAPP-β	Soluble ectodomain β
TBI	Traumatic brain injury
TBS	Tris-buffered saline
TBST	TBS + 0.1% Tween-20
TKO	Tau knockout
WT	Wild type

Chapter 1

Introduction

1. A Brief History of Alzheimer's disease and dementia

Dementia in humans has been recognized since at least 2000 BC. It was first recorded by ancient Egyptians who correlated old age to advancing memory loss, although interestingly, they believed that the heart and the diaphragm controlled a healthy mental life. In Greek and Roman histories as well, there are documented instances of bizarre behavior by famed historic figures in old age (Boller and Forbes, 1998). The philosopher Plato postulated that old age was always accompanied by dementia, although later contemporaries pointed out that dementia was not necessarily a consequence of growing old (Boller and Forbes, 1998). Into the early common era ages and up to the 18th century, various associations between old age and cognitive decline were noted by histeorical figures. Pythagoras, the famous mathematician and philosopher, classified humans into six categories based on age: the later two, called senescence (for those aged 63-79) and old age (age 80 and older), he regarded as a declining phase of body and mind, with some of those in old age showing mental degeneration (H. D. Yang et al., 2016). It wasn't until 1797, however, that a formal and detailed description of dementia existed. This definition came from the studies of the founder of modern psychiatry Philippe Pinel. Pinel's contemporary, Jean Etienne Esquirol, followed up this definition with the following description: "Dementia is that disabilities are shown in discernment, intellectual ability and will due to brain diseases, and is to lose joyfulness enjoyed and is that the rich become poor." (Torack, 1983; H. D. Yang et al., 2016).

It wasn't until 1907 in Germany, with the case of Auguste Deter, that a clinically distinct form of dementia was first described. The physician Alois Alzheimer noted that this patient's symptoms began with memory loss and disorientation, and over the course

of five years, the patient progressed into depression, hallucinations, and eventually dementia and death. He correlated these clinical symptoms with atrophy and lesions in the cerebral cortex, and large distorted neurofibrils we now know as the two hallmark lesions of the disease: senile plaques and neurofibrillary tangles (NFT; Holstein, 1997). In 1910, Alzheimer's colleague, Emil Kraepelin, published the first discovered instances of this form of dementia using the name Alzheimer to categorize the disease (Hippius and Neundörfer, 2003).

Since the discovery of AD in 1907, the prevalence of AD has progressed significantly, with Alzheimer's disease being the most common form of dementia, and the sixth leading cause of death as of 2018. The following section provides a detailed description of this disease, and follows the many aspects of its research done over the course of the 20th and 21st centuries.

2. Current state of AD diagnosis:

As the French medical writer Jean Etienne Esquirol writes about patients with dementia: "a demented man has lost the goods he used to enjoy; he is a wealthy person turned poor" (Esquirol, 1838). This statement, true figuratively when it was written in 1838, is true quite literally today: Alzheimer's patients in the United States cost the nation $259 billion dollars in 2017, and with the aging population increasing in size, this number is projected to reach $1.1 trillion dollars by 2050 (Alzheimer's Association, 2017). AD is the most common form of dementia and the sixth most prevalent disease in the United States, but its costs surpass those associated with both heart disease and cancer. Worse still, AD is the only disease in the top ten most common disorders that still

has no disease-modifying treatments available (Citron, 2010; Cummings, Morstorf and Zhong, 2014). Recently, many clinical studies have been done using antibody therapies to amyloid-β (Aβ), but many of these trials are unsuccessful, and a curative treatment remains elusive (Karran and Hardy, 2014; Hardy and De Strooper, 2017). Of the current drugs used to treat AD, three are acetylcholinesterase inhibitors, thought to prolong synaptic function (Kaduszkiewicz et al., 2005), and an N-methyl-D-aspartate receptor (NMDAR) antagonist, memantine, that blocks excess calcium influx through the receptor while maintaining normal synaptic transmission (Lipton, 2006).

Current official diagnosis of AD is not made until postmortem, when a patient's brain can be examined for plaques and tangles (American Psychiatric Association and Force, 2013). However, clinicians can make a diagnosis of probable AD using a variety of cognitive tests. Patients are diagnosed on a spectrum, beginning at the pre-diagnostic AD stage where no clinical symptoms are present, but damage has occurred at the level of individual neurons. Progression of the disease leads to mild cognitive impairment due to AD, and then through the mild-, moderate-, and severe dementia related to AD stages. The pre-diagnostic stage is of special interest, as this stage can last 20 years or longer before patients proceed to the more detrimental stages, making it the best opportunity for therapeutic intervention (Sperling et al., 2011). Each of these stages is unique in terms of the biomarkers that appear and the progression of decline in the patient's cognitive state.

Recently developed guidelines published by a joint meeting of the National Institute of Aging and the Alzheimer's Association are available for assessing patients for a probable Alzheimer's diagnosis (Albert et al., 2011; Dubois et al., 2016). These guidelines include longitudinal studies of a patient's cognitive abilities over time,

including memory, problem solving, and attention span, as well as testimony from an objective representative, such as family or caretakers (Albert et al., 2011). In addition to assessing a patient's cognitive abilities through consultation, procedures such as CT and MRI can be used to assess structural damage or atrophy, scan for biomarkers and to rule out cognitive disabilities not related to AD, such as ischemia or stroke (Albert et al., 2004; Hyman et al., 2012).

In addition to using these procedures on their own, MRI and PET scans can also be coupled with different contrast dyes to assess aggregate load in the brain. Among the most prominently used dyes is Pittsburgh Compound B (PiB), which binds specifically to Aβ fibrils allowing plaque load to be visualized in the living brain (Klunk et al., 2004). Current research has also established dyes that allow for visualization of tau in the brain (Okamura et al., 2014; Bischof et al., 2017). In addition to amyloid and tau plaque loads being characteristic of neurodegenerative diseases, another marker of decline in AD is hypometabolism in the brain, which can be measured by using PET scans coupled with fludeoxyglucose to measure metabolic activity of neurons (Mosconi et al., 2008). These diagnostic tools are useful not only in tracking structural and pathological changes in the brain over time in individual patients, but are also helpful for determining if patients are eligible for clinical trials of putative AD therapies. This would provide the opportunity to diagnose patients before they show signs of cognitive impairment.

Although MRI and PET scans are great diagnostic tools, there are problems remaining with them: first and foremost is that these tools are expensive, and in the case of PET, not widely available. Second is being able to diagnose patients early enough such that intervention with putative AD drugs happens early enough to be disease altering.

Screening for biomarkers in CSF, blood, saliva, or urine samples from patients could streamline diagnosis, as well as finding suitable candidates for initial drug trials (Humpel, 2011). Using CSF to detect levels of amyloid beta and tau is the one of the best methods currently used to diagnose patients before significant damage to the brain occurs, although it is still an invasive procedure requiring hospitalization and carrying risks of spinal cord injury and infection. Still, CSF biomarker screens are a valuable tool for both current disease treatments and for studying any potential disease-modifying therapies (Blennow and Hampel, 2003). In addition to CSF biomarkers, there are some detection methods being developed to detect amyloid levels in blood plasma by mass spectrometry (Ovod et al., 2017), however these methods are still in development and are not yet widely used for diagnostics.

Many of the current biomarker studies use amyloid readout as a measure of disease progression by looking at PiB-PET scans in combination with screens for levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the CSF. These forms of Aβ are the first to accumulate in the CSF, before other proteins appear and before neurological symptoms present (Forsberg et al., 2008; Grimmer et al., 2009; Buchhave et al., 2012). Clearance of $A\beta_{1-40}$ and $A\beta_{1-42}$ through the CSF decreases over the course of disease progression compared to cognitively normal individuals (Mawuenyega et al., 2010), and this decrease in amyloid CSF correlates to increases in amyloid visualized by PiB-PET imaging (Fagan et al., 2006). In addition to looking at Aβ levels in the CSF, levels of total tau and phospho-tau can be quantified and used as an additional correlate of cognitive decline in AD, although seemingly not in tauopathies that lack amyloid pathology (Jack et al., 2011). Tau accumulates in the CSF after Aβ is detected, with the levels of tau increasing over the course of disease

progression, but still before major neurological symptoms appear. There are groups currently working on coupling tau PET imaging to screening for total and phospho-tau levels in the CSF as an additional method for screening for disease progression (Gao, M. Wang and Q.-H. Zheng, 2015; K. A. Johnson et al., 2015), however, these tau dyes are still early in development.

3. Risk Factors for AD

Although AD is one of the most prevalent diseases in existence, its root cause remains unknown. Despite not understanding how each individual case of AD begins, there are known risk factors that increase susceptibility to the disease. The first and most prominent risk factor associated with AD is age: with the exception of AD cases where a familial mutation is present, the symptoms of sporadic AD first present clinically around age 65. Those between the age of 65-74 have a 3% chance for developing AD, and from there this figure rises dramatically, with the risk of developing AD rising to almost 50% in those 85 and older (Alzheimer's Association, 2017). It seems that with AD, there are no inherent sociological biases of the disease, i.e. disease onset is not more common among a certain race or gender. Rather, perceived biases in disease onset come from differences in socioeconomic factors such as education (Sattler et al., 2002; Karp, 2004) and lifestyle differences (Norton et al., 2012; Tolppanen et al., 2015), or follow differences in demographics already occurring in a particular population (e.g. more women than men suffer from AD in the United States, but women are also living longer).

Alzheimer’s disease occurs in one of two forms: an early-onset form that occurs due to familial mutations, long before the age of 65, and a late-onset, sporadic form that

occurs in patients from 65 years of age and beyond, and has no determinable root cause. The majority of cases of AD that occur are both late-onset and sporadic, meaning there are no familial mutations causing presentation of the disease. In these instances, there is no one causal factor that can be determined. A more recently discovered genetic risk factor associated with the sporadic form of AD is for the APOE gene. This gene encodes a cholesterol binding protein that is thought to aide in the clearance of amyloid from the brain, thus influencing the rate of Aβ accumulation in brain. The APOE gene has three alleles, ε2, ε3, and ε4. Individuals with an ε4 allele are at an increased risk for developing AD, and those with two ε4 alleles are at a higher risk than those with only one copy (Corder et al., 1993; Genin et al., 2011). It has been shown that the ε4 allele binds to amyloid less tightly than other isoforms (Strittmatter et al., 1993), and could be less effective in clearing amyloid. Thus, a theory for the APOE risk factor is that having this isoform leads to less clearance of amyloid from the brain, thus contributing to its the accumulation. Conversely, individuals that have one or two ε2 alleles show a decreased risk for the disease, and the frequency of this allele is decreased among those who have or had AD (Corder et al., 1994).

In addition to the genetic predispositions that increase susceptibility to AD, there are modifiable risk factors that also play a role (Reitz and Mayeux, 2014). In line with glucose and metabolism signaling being altered in individual neurons, obesity and type II diabetes both increase the likelihood of a person getting AD (Luchsinger et al., 2001; Xu et al., 2011). The role of these risk factors in AD is also summarized as the “type III diabetes” model of AD, due to the role that insulin resistance has in oxidative stress and cognitive impairment in the brain, along with signaling changes at the level of the

individual neuron (la Monte, 2014). Heart disease, hypertension, low physical activity and smoking also contribute to one's risk of developing AD (Lindsay, 2002; Luchsinger et al., 2005). In addition to the risk of each of these factors on their own, they can compound one another, such that having multiple risk factors associated with an individual further increases their risk of getting the disease. Individuals who have suffered traumatic brain injury (TBI), such as concussions, are also at an increased risk for AD (Mortimer et al., 1991; Y. Li et al., 2017). It is important to recognize that these risk factors show correlations with increased instance of AD, rather than direct causation of the disease. It is likely that in these sporadic cases there are combinations of risk factors making people susceptible to AD, rather than one root cause to blame. In addition to lifestyle factors that increase susceptibility to AD, there are some factors that show a negative correlation with disease instance. These factors include regular exercise (Radak et al., 2010; Ahlskog et al., 2011) and use of non-steroidal anti-inflammatory drugs (NSAIDs; P. L. McGeer and E. G. McGeer, 2007).

There are also a small number of familial mutations which significantly increase one's risk for AD. These familial forms differ in that they are fully penetrant for AD, and that generally patients present with symptoms before the age of 65, hence characterizing this form as early-onset. The number of familial cases of AD pales in comparison to the number of sporadic cases: it is generally accepted that less than 1% of total AD cases can be traced back to a causal genetic mutation (Bekris et al., 2010). Other studies looking more broadly at early-onset AD that is not necessarily linked to a genetic mutation report a number between 1-6% of total AD cases (Citron et al., 1997; Bekris et al., 2010; Reitz

and Mayeux, 2014; X.-C. Zhu et al., 2015). Although these cases are sparse relative to total AD cases, they present similarly to sporadic forms of the disease.

The mutations that cause early-onset AD provided some of the first insights into how the disease manifests. The genetic mutations that make up familial forms of AD occur in the genes for amyloid precursor protein (APP), as well as presenilins 1 and 2, which play a role in APP processing and amyloid production (Selkoe, 1997). The specific mutations, as well as how they affect amyloid production, will be discussed in greater detail in the APP section of this chapter. Also relevant to APP is the instance of AD in Down syndrome patients. The gene for APP is located on chromosome 21, meaning those with Down syndrome have an extra copy of the gene and produce more Aβ. Most individuals with Down syndrome develop plaques and tangles, and many show signs of the cognitive decline associated with AD as they become older (Mann, 1988; Chapman and Hesketh, 2000; Holland et al., 2000).

4. Neurophysiological symptoms of AD

How Alzheimer's disease manifests initially is still an active field of research. The defining characteristics of the disease are two types of protein aggregates in the brain: extracellular senile plaques containing amyloid beta, and neurofibrillary tangles contained within the neuron. Although there are many negative consequences on neurons caused by amyloid beta and tau at the cellular and molecular levels, much of the damage contributing to neuron dysfunction and death can be traced back to the loss of synapses and synaptic function (Koffie, Hyman and Spires-Jones, 2011).

Loss of synapses is the best correlate of disease progression in Alzheimer's to date, though it is hard to quantify this loss clinically or before an autopsy is performed (Terry et al., 1991). Major synaptic dysfunction and neuron loss in AD occurs first along the entorhinal cortex and perforant pathway of the hippocampus, where sensory information is received and processed, and later in the neocortex and cortical areas of the frontal, temporal, and parietal lobes (Price et al., 1991; Mattson, 2004). Many of the neurons damaged in AD are excitatory, which results in the loss of NMDA- and α-amino-methyl-propanoic Acid (AMPA)-containing neurons in the brain as the disease progresses. Additionally, as these areas of the brain are the major points for sensory input and processing, as well as retention and recollection, it makes sense that the major symptoms of AD are memory loss and cognitive decline.

By the time an Alzheimer's diagnosis is made, there is extremely significant synapse loss and neuron death. However, research shows that the biological changes in the brain that eventually lead to an Alzheimer's diagnosis can occur years or even up to decades before the disease fully manifests. Many of the initial changes in the brains of AD patients occur at the level of the individual neuron (De Strooper and Karran, 2016). In addition to problems occurring at the synapse, there are many changes occurring within the neuronal body causing dysfunction and death. These changes include mitochondrial dysfunction, leading to the production of radical oxygen species (ROS) and substantial oxidative stress that signals for death, as well as changes in glucose uptake and subsequent metabolism signaling via mTOR (Miranda et al., 2000; Lin and Beal, 2006). There are also changes in the levels of calcium from various sources, including extracellular calcium entering through the NMDA receptor and intracellular calcium from

the ER and mitochondria (Workgroup1, 2017). This excess calcium leads to changes to where and when calcium signaling takes place, oftentimes erroneously, leading to excitotoxicity that contributes to the eventual death of the neuron. Many neurons will attempt to re-enter the cell cycle aberrantly in AD, particularly those in cortical and hippocampal regions of the brain that are susceptible to other insults, marking neurons that are in danger early in disease progression (Herrup and Y. Yang, 2007a; H.-G. Lee et al., 2009).

Many of the symptoms of dysfunctional neurons mentioned so far are directly related to exposure to Aβ oligomers (AβOs). Although there are many symptoms at the level of neurons that occur due to exposure to AβOs, tau also plays a significant role in the demise of neurons in AD (Medeiros, Baglietto-Vargas and LaFerla, 2010). The microtubule-associated protein (MAP) tau is primarily an axonal protein, and its main functions include the stabilization of microtubules and regulation of transport along the axon. Eventually in AD, tau accumulates to form oligomers and eventually large insoluble tangles that encompass much of the volume of the neuron. However, much like AβOs versus larger plaque accumulation, it seems that tau is most damaging in its soluble form, namely in variously post-translationally modified monomers and oligomers (Kopeikina, Hyman and Spires-Jones, 2012). Included in the various changes that occur to a neuron in AD is aberrant activation of many kinases, some of which phosphorylate tau. This phosphorylation, as well as other post-translational modifications, lead to many changes in tau, including its dissociation from the microtubule (Iqbal et al., 2010). These changes in tau lead to disruptions in trafficking along the axon, as well as mislocalization

of tau to the somatodendritic compartment, where tau can recruit different signaling molecules, or even act as a signaling molecule in its own right.

In subsequent sections, I will detail these individual symptoms and how they interact to cause neuron death. What is important to recognize, not only in AD but in many disease paradigms, is that there is no one molecular malfunction leading to the death of neurons in AD. Rather, it is the culmination of individual insults at the molecular level over time, compounding their effects on individual neurons and resulting in the neuronal dysfunction and eventual death seen in AD. This makes the disease extraordinarily difficult to combat.

5. APP

The amyloid precursor protein (APP) is a type I single-pass transmembrane protein that is expressed in most cell types, but is especially abundant in neurons. It is part of a small gene family of transmembrane proteins called the amyloid precursor protein like family that includes APP and the proteins APP-like (APPL) 1 and 2 in humans (O'Brien and Wong, 2011). The APPL family of genes all contain long extracellular domains with short cytoplasmic tails. These proteins also have a number of domains in common, including a heparin-binding domain and copper-binding domain contained within the extracellular domain, which together compose the E1 domain. The E1 domain is immediately followed by an acidic domain, and then an E2 domain, also contained in the extracellular domain. At the C-terminus of the APPL gene family proteins there is a YENPTY domain that is conserved throughout multiple species and that controls the intracellular sorting of APP via clathrin-

mediated internalization (W. J. Chen, Goldstein and Brown, 1990). The Aβ domain is unique to APP, with the sequence differing significantly in the APPL proteins (Muller and H. Zheng, 2012). The sequence that becomes Aβ upon APP processing occurs where APP passes through the cell surface membrane. The N-terminus part of the Aβ sequence located in the extracellular space and the C-terminus part of the sequence contained within the membrane, demonstrating the amphiphilic nature of Aβ.

A number of functions have been postulated for APP based on its various domains, though widely acknowledged functions for APP remain elusive (Thinakaran and Koo, 2008). It is postulated that APP is an essential protein for brain development, and that the secreted form of APP (sAPP) has a possible role in synaptic plasticity, as expression of APPs or injection of the peptide in mice led to increased synaptic density and better memory retention (Roch et al., 1994; Meziane et al., 1998; Thinakaran and Koo, 2008). It is also thought that APP plays a role in cell adhesion (Breen, Bruce and Anderton, 1991; Ghiso et al., 1992) and regulates neurite outgrowth during development (D. H. Small et al., 1994).

Although there are many putative roles for APP in normal neuron development and function, mouse models with APP constitutively knocked out are viable, displaying only minor deficiencies, indicating APP on its own is not critical. Specifically, these mice have decreased locomotor function as demonstrated by grip strength, and they show signs of reactive gliosis by 14 weeks (H. Zheng et al., 1995), indicative of impaired neuronal function. Additionally, these mice show hypersensitivity to kainic acid induced seizures (Steinbach et al., 1998). It is thought

that the proteins APPL 1 and 2 have redundant roles to APP, and can make up for the loss of function in the APP knockout mice (APP-KO).

Similar to APP-KO mice, single KO mice deficient in either APPL-1 or APP-2 are viable, with APPL-1 deficient mice showing mild functional deficits (Heber et al., 2000) and APPL-2 deficient mice showing no apparent complications (Koch et al., 1997; Muller and H. Zheng, 2012). However, some APP gene family double knockouts showed more serious complications: mice lacking either APPL-1 and APPL-2, or APP and APPL-2, die shortly after birth, but interestingly, APP/APPL-1 knockout mice are viable and relatively healthy (Heber et al., 2000). This implicates a distinct physiological role for APPL-2 in addition to its postulated compensatory role for APP. As is expected, triple APP-family knockouts die shortly after birth. The brains of the triple KO embryos differ from the two lethal double KO mice though, in that many of the triple KOs show structural damage in the embryonic brain, whereas there are no overt histological abnormalities in either of the lethal double KO combinations (Herms et al., 2004).

There are various changes that occur to APP as it goes through the secretory pathway. As APP moves through the ER and the trans Golgi network, it can be N- and O-glycosylated, phosphorylated, and tyrosine sulfated before reaching the plasma membrane. From the plasma membrane, APP is quickly internalized, moving through the endocytic pathway, where it is either degraded or sent back to the cell surface. Throughout its time at the cell surface and in the endocytic pathway, APP can be processed by enzymes known as secretases, generating various cleavage products,

including Aβ. Where these cleavage products end up largely depends on where they are processed (Haass et al., 2012).

APP can be processed via one of two main pathways centered around the production of Aβ: the amyloidogenic and non-amyloidogenic pathways (De Strooper, Vassar and Golde, 2010; See Figure 1). The non-amyloidogenic pathway is the predominant APP processing pathway in healthy individuals. In this processing pathway, α-secretases at the cell surface cleave APP, forming two byproducts: a soluble APP-α ectodomain (sAPPα) fragment and an 83 amino acid C-terminal fragment (CTF) that stays at the cell surface. The α-secretase cut site on APP occurs within the region of APP where Aβ originates, thus precluding the formation of Aβ. The α-CTF left in the membrane can be further processed by γ-secretases, producing a P3 N-terminally fragmented Aβ protein, and the APP intracellular domain (AICD). There has been no role attributed to the P3 fragment, and it appears to be an innocuous byproduct. The AICD has been shown to be involved in multiple signaling pathways (Reinhard, Hébert and De Strooper, 2005), including phosphoinositide-mediated calcium signaling (Leissring et al., 2002; Kinoshita et al., 2002), and thought to be involved in transcriptional regulation (Pardossi-Piquard et al., 2005), though this is contested by some (Cao and Südhof, 2004; A. C. Chen and Selkoe, 2007).

APP processing via the amyloidogenic pathway begins with APP being cleaved by the β-secretase BACE1, which is also the rate-limiting step of Aβ formation (Vassar et al., 1999). BACE1 is localized specifically to late Golgi and endosomal compartments (Yan et al., 2001), meaning the first cleavage event occurs after full

length APP is internalized from the cell membrane via endocytosis. Cleavage of APP by BACE1 generates the soluble ectodomain fragment sAPPβ, and a 99 amino acid CTF containing the Aβ sequence (Vassar et al., 1999). This β-CTF fragment is then processed by γ-secretase, generating an Aβ peptide. Most γ-secretase activity is seen at the plasma membrane, though a small amount of activity can be seen in endosomes (S. A. Small and Gandy, 2006; Kaether et al., 2006), which is in line with the generation of extracellular and intracellular Aβ, respectively. Unlike the precise α and β secretase cleavage sites, the γ secretase cut site is less specific, generating both 40- and 42-residue varieties of the Aβ peptide (Kakuda et al., 2006). It is unclear what changes occur to bias APP toward one processing pathway over the other, barring the familial mutations discussed in the next paragraph.

There are a number of familial mutations that occur in APP, as well as in the subunits presenilin-1 and 2 of γ-secretases. These mutations can bias the cleavage of APP toward the amyloidogenic pathway, generating more Aβ that accumulates over time, or lead to an increase in the Aβ42: Aβ40 ratio. As mentioned previously, these mutations are fully penetrant for AD, and symptoms in these familial early-onset AD patients can be evident by the time they reach their 30's. According to the Alzheimer's Association, there are currently than 26 different pathogenic mutations in APP that that lead to toxic Aβ production (Alzheimer's Association, 2018). These mutations either occur near the β- and γ-cleavage sites increasing Aβ levels directly, or at sites making the non-amyloidogenic cleavage more difficult, with both instances leading to an increase in the production of toxic forms of Aβ. These mutations were first discovered in family cohorts with increased instances of early onset AD, and are

named for the areas where these families lived (i.e. the K670M/N671L APP mutation is referred to as the Swedish mutation, as this is where it was first discovered). Interestingly, there is one known APP mutation that is protective against AD, known as the Icelandic mutation (Jonsson et al., 2012). This mutation occurs in APP very close to the β-secretase cut site, and is thought to prevent the β cleavage, thus preventing Aβ production. It has been shown that Aβ derived from this APP mutant isn't less toxic, but rather that less Aβ is being produced in these instances (Maloney et al., 2014).

The majority of mutations that involve γ-secretase occur in presenilin-1, with the Alzheimer's Association noting over 180 known PSEN1 mutations to date, and 15 known pathogenic PSEN2 mutations that cause early onset AD (Alzheimer's Association, 2018). All PSEN mutations act to increase the production of Aβ, similar to familial APP mutations. In addition to increased total amyloid levels, some familial mutations associated with AD, namely the London and French mutations in APP, increase the Aβ42/Aβ40 ratio, and Aβ42 has been shown to be a more toxic variety of the Aβ peptide (Scheuner et al., 1996; Citron et al., 1997). These mutations give support to the amyloid cascade hypothesis, which states that AD initiates from Aβ formation, with all other symptoms of the disease stemming from Aβ production and accumulation (Hardy and Higgins, 1992; Selkoe and Hardy, 2016).

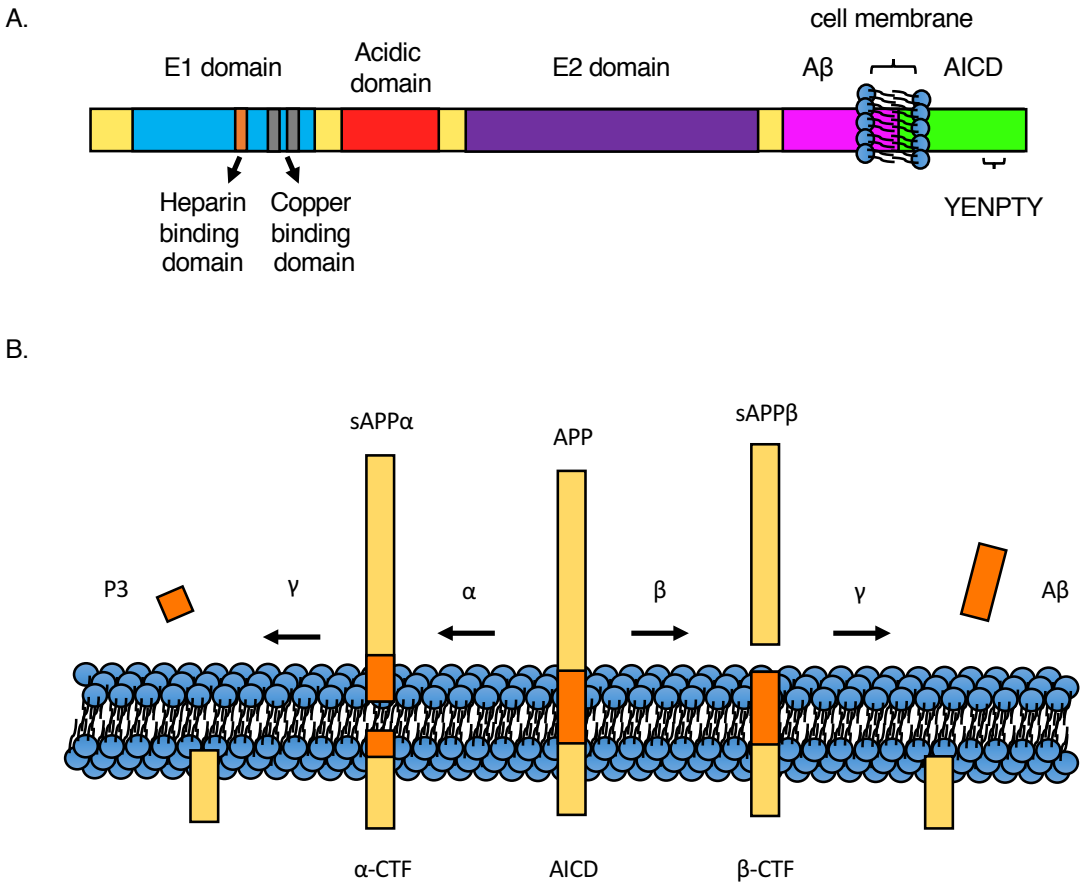


Figure 1: APP Processing pathways. Adapted from De Strooper, Vassar and Golde, 2010, and Evin and Li, 2012. The amyloid precursor protein can be processed by one of two pathways: the non-amyloidogenic or amyloidogenic pathway. In the non-amyloidogenic pathway, alpha secretase cleaves through the sequence that would become Aβ, creating a soluble APP-α fragment that can be further processed by γ-secretase. In the amyloidogenic pathway, which results in Aβ, APP is cleaved by the β-secretase BACE1, creating a similar soluble APP fragment which is subsequently processed by γ-secretase, producing Aβ.

6. Aβ (general/structure)

Aβ peptides have been heavily discussed and debated for their role in AD pathology for decades. Existing predominantly in the extracellular space, and the first protein known to aggregate in AD, Aβ has been long known to play a significant role in the initiation and continuing pathology of AD. The association between plaques and dementia has been known since the first description of the disease in 1907. Since this time, it has been discovered that plaques are made primarily of the Aβ peptide, aggregated into insoluble masses by the time of significant disease progression. These plaques are found in the extracellular space, particularly in areas with high levels of dystrophic neurons and morphological abnormalities, such as the limbic and association cortices (Hyman et al., 1984; Rogers and Morrison, 1985). Structurally, plaques are made up of Aβ fibrils arranged in β-sheets, and can range in size from ~1500 to ~2500 μm^2 mean cross-sectional area (Rogers and Morrison, 1985). The discovery of variously sized Aβ peptides making up senile plaques was the first connection to familial AD mutations and disease onset made, beginning decades of intense research into how Aβ perpetuates AD, and the attendant dysfunction and death of neurons. The following section will discuss the structure and functions of Aβ as they relate to AD.

As discussed in the APP section, Aβ peptide comes from the cleavage of APP by β- and γ-secretases, expelling Aβ into the extracellular space or lumens of intracellular vesicles. Aβ peptides ranging in length from 16-43 amino acids have been found *in vivo*, due to variability at the β-secretase and more commonly the γ-secretase cut sites (De Strooper, Vassar and Golde, 2010), and additional proteases that trim the initial products of APP hydrolysis by β- and γ-secretases. The dominant species found throughout

plaques are $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, with the numbers corresponding to amino acid length (Thinakaran and Koo, 2008). These Aβ peptides are also found with various post-translational modifications, such as pyroglutamylation at the third or eleventh residue, forming an Aβ peptides such as $A\beta_{3pE\text{-}40}$, $A\beta_{3pE\text{-}42}$, $A\beta_{11pE\text{-}40}$, and $A\beta_{11pE\text{-}42}$ (Jawhar, Wirths and Bayer, 2011). This post-translational modification, particularly the $A\beta_{3pE\text{-}42}$ variety, has been shown to greatly increase the toxicity of Aβ to cultured neurons and in mouse brain, and is thought to be an initiator of oligomer formation and toxicity in AD (Schilling et al., 2008; Nussbaum et al., 2012). Additionally, it has been shown that increases in the ratio of $A\beta_{1\text{-}42}$ to $A\beta_{1\text{-}40}$ increases the propensity of Aβ to aggregate, also contributing to increased toxicity of the peptide (Pauwels et al., 2012).

Aβ peptides are able to aggregate into soluble, low-n oligomers such as dimers and trimers up through larger soluble species, such as dodecamers, and finally into larger insoluble fibrils, which accumulate in plaques. Although initially it was believed that fibrils were the disease-propagating species in AD, there is now substantial evidence that the species involved in Alzheimer's pathology are the soluble oligomers (Cleary et al., 2005; Haass and Selkoe, 2007). The first evidence for this came after the discovery of the Aβ peptide, when it was found that Aβ monomers were non-toxic to cultured hippocampal neurons, but that allowing the peptide to incubate in solution for days caused the peptide to aggregate, making it neurotoxic (Pike et al., 1991). More evidence supporting the role of AβOs in AD progression came from the observation that although large plaques could be seen around areas of extreme neuronal damage, there was no correlation between plaque load and the onset of behavioral symptoms of AD (McLean et al., 1999; J. Wang et al., 1999). Rather, a much better correlate to symptom onset is Aβ

oligomer levels, suggesting that plaques are not toxic *per se*, but rather they are a consequence of Aβ accumulation over time. In line with this, there are examples of AD patients who have little plaque load despite extensive neuron damage, and 'healthy' individuals who are found to have numerous plaques but no neuronal damage, suggesting that plaque deposition in itself is not toxic (Herrup, 2015). Further evidence of this comes from a study in AD mice showing that surrounding Aβ plaques are halos of soluble oligomeric species, and that the majority of synapse loss occurring near these plaques overlaps with these AβO halos (Koffie et al., 2009). This study also shows that in brain areas affected by AD where neurons are at least 50 μm from a plaque, synapse density reaches WT control levels. The implications of this study are that AβOs specifically are contributing to synapse loss, and that plaques are acting more as AβO sinks rather than the toxic species themselves.

There are also numerous published reports of interactions of AβOs with receptors at the cell surface of neurons, specifically at the post-synapse (Patel and Jhamandas, 2012). One of the first Aβ-receptor interactions to be discovered was with the α7 acetylcholine receptor (H. Y. H. Wang et al., 2000), a finding that supports the cholinergic hypothesis of AD. This hypothesis, first described in 1982, states that loss of cholinergic synaptic transmission and eventually of cholinergic neurons contributes significantly to the cognitive decline of AD (Bartus et al., 1982). Over time, there have been numerous other interactions of AβOs with different receptors, including the NMDA receptor (Lacor et al., 2007; De Felice et al., 2007) and metabotropic glutamate receptors (Rammes et al., 2011), β2 adrenergic receptor (D. D. Wang et al., 2010), insulin receptor (Xie et al., 2002; W.-Q. Zhao et al., 2008), prion protein (Um et al., 2012), and the p75

neurotrophin receptor (Yaar et al., 1997; Patel and Jhamandas, 2012). Each of these interactions can induce various signaling pathways contributing the the overall dysfunction of the neuron.

All of this evidence culminates into what is known as The Amyloid Hypothesis of Alzheimer's disease (Hardy and Higgins, 1992; Selkoe and Hardy, 2016). In its latest iteration, the hypothesis states that in AD, Aβ accumulates due to an increased rate of production and/or a decreased rate of clearance, and that over time, this Aβ forms soluble oligomers and protofibrils, eventually forming the plaques that are characteristic of the disease. Furthermore, it states that Aβ is the initiator of AD, with all subsequent neuronal insults such as oxidative stress, calcium and synapse dysfunction, and tau aggregation stemming from this Aβ accumulation. The first evidence for this hypothesis came from the familial mutations fully penetrant for AD: all of the mutations occur either to bias APP cleavage toward Aβ production, or in the γ-secretase subunit presenilin that cleaves Aβ from the sAPPβ fragment.

Over time, extensive research has been done that supports the idea that AβOs initiate much of the neuronal damage occurring in AD, particularly at the molecular level (McLean et al., 1999; Haass and Selkoe, 2007; Sakono and Zako, 2010). However, there are some problems raised with the idea that Aβ is the sole perpetuator of AD: namely, that plaques can exist in healthy individuals without causing neuronal deficits, and that despite numerous AD mouse models that overexpress APP and generate AβOs and plaques, these models rarely show tau pathology or overt neuron death (Herrup, 2015). These counterpoints do not seem to contend with the idea that Aβ is acutely involved in AD pathology. Rather, it seems as though AβOs are just one causative agent in the

disease, and there are numerous dysfunctions occurring simultaneously, with the eventual consequence of neuron death in AD. Supporting these counterpoints further is the fact that tau has also been shown to be necessary for the etiology of AD, and that eliminating tau ameliorates many neuronal deficits, such as sensitivity to excitotoxicity, learning and memory deficits (Roberson et al., 2007), and re-entry into the cell cycle (Seward et al., 2013).

Even within the oligomeric species of Aβ, there is much debate about what denotes a species as toxic, and which types of oligomers are responsible for the various insults to neurons. Preparation of oligomers can vary widely depending on factors such as starting material, pH and salt concentrations, temperature, or duration of oligomerization. Furthermore, the amphiphilic nature of the Aβ peptide allows it to adopt a heterogeneous range of conformations in solution that likely exist along an equilibrium between various states of oligomerization (Benilova, Karran and De Strooper, 2012). Furthermore, it is often difficult to isolate discrete populations of oligomer, as this is often influenced by the analytical method used to measure a given sample. For example, it has been shown that analysis of Aβ peptide by SDS-PAGE can both artificially induce oligomer formation of monomeric peptide, and break down larger aggregates of amyloid to show the appearance of low-n oligomers in what should be fibrillar preparations (Bitan et al., 2005). In another study, it was found that when an AβO preparation was run on size exclusion chromatography, there was a discrete population of AβO species running at approximately 65-80 kDa, indicative of a dodecamer. When this same ‘dodecamer’ SEC fraction was analyzed by multi-angle light scattering, however, it was found to have molecular weight species ranging from 150-1,000 kDa (Hepler et al., 2006). Further

complicating AβOs is difference in potency between human derived and synthetic preparations of AβOs. Whereas oligomers purified from brain are bioactive at high picomolar to low nanomolar concentrations, synethetic preparations of AβOs are typically used at high nanomolar to low micromolar concentrations to see similar affects (Selkoe, 2008). This suggests that structure and post-translational modifications that may occur with human AβOs play an equally if not more important role to oligomer size in amyloid toxicity.

7. Tau

The protein tau also plays a critical role in AD pathology and is the major component of neurofibrillary tangles found within neurons of AD brain. Although AD is the most prevalent disease involving tau, insoluble aggregates of tau in the brain are involved in numerous disorders, collectively known as tauopathies. Tau was initially characterized as a microtubule-associated protein involved in facilitating the formation of microtubules and aiding in maintaining their stability (Witman et al., 1976). It is expressed predominantly in the axons of neurons (Binder, Frankfurter and Rebhun, 1985). Tau is extremely heat stable and hydrophilic, making it very soluble (Cleveland, Hwo and Kirschner, 1977; Grundke-Iqbal et al., 1986). In its native state, tau is not prone to aggregation, though hyperphosphorylation and dissociation from the microtubule can facilitate its aggregation into insoluble paired helical filaments (PHF) that make up the tangles found in AD brain. In the human brain there are six isoforms of tau, each of which is produced through alternative spicing of the MAPT gene on chromosome 17. Tau is characterized by four types of domains: an N-terminal repeat domain, a proline-rich

domain at the N-terminus, a microtubule binding domain (MTBD) containing 3 or 4 tandem, imperfect repeats, and a C-terminal domain (Ballatore, V. M. Y. Lee and Trojanowski, 2007) (See Figure 2). The six isoforms are named based on the number of inserts contained in the N-terminal domain and the number of repeats in the MTBD, thus the six isoforms are 0N3R, 0N4R, 1N3R, 1N4R, 2N3R, and 2N4R, with 0N3R and 2N4R being the shortest and longest isoforms, respectively (Goedert, Crowther and Garner, 1991).

Tau in its native state is an unstructured protein, though it becomes more tightly folded upon binding to microtubules. Despite having no secondary structure, in solution tau has been observed to adopt what is known as a paperclip conformation, where the N- and C-termini are in close proximity to one another. This conformation has also been shown to become tighter or looser depending on the phosphorylation state of the protein, giving insight into how it becomes pathogenic upon phosphorylation (Jeganathan et al., 2008).

Overall, tau is a slightly basic protein, with regions that are either neutral or acidic, which are important for interactions with binding partners, as well as for its folding and aggregation (Y. Wang and Mandelkow, 2015). The N-terminal domain of the protein is negatively charged, and increasingly acidic based on the number of N-terminal inserts. The proline rich region of tau is positively charged, and thus is where a number of the phosphorylation sites on tau occur (Y. Wang and Mandelkow, 2015). Additionally, this region contains seven PXXP motifs that bind Src-homology 3 (SH3) domains on other proteins, most notably the src-family kinase Fyn, that has been shown to both bind tau and phosphorylate it at its Y18 and Y29 residues (G. Lee et al., 1998; 2004). These

two regions together are also referred to as the projection domain, as they project from the microtubule upon binding. Furthermore, they can determine the spacing between microtubules within a microtubule bundle (J. Chen et al., 1992). The MTBD toward the C-terminal of tau has a series of 3 or 4 imperfect repeats that consist of stretches of 18 residue regions that are highly conserved, followed by 13- or 14-residue linker regions that are less homologous (Gustke et al., 1994). This region also facilitates tau binding to lipid membranes, which can facilitate their aggregation (Georgieva et al., 2014). As expected, tau containing 4 of these domains (4R tau) binds more tightly to microtubules than 3R tau does. Like the projection domain, the C-terminal domain of tau also sticks out from the microtubule when bound, and contains neutral and acidic subdomains that indirectly regulate microtubule binding (Buée et al., 2000).

There are a number of post-translational modifications that can occur on tau, including glycation, deamidation, prolyl-isomerization, nitration, methylation, ubiquitylation, and sumoylation (Martin, Latypova and Terro, 2011), though the most notable in terms of disease pathogenesis is phosphorylation. Tau can be found phosphorylated at a low level in healthy brains, with an average of 2 phosphorylation sites per molecule in adult brain. In many neurodegenerative disorders, however, tau becomes hyperphosphorylated, with an average of 8 phosphorylations per molecule, partially due to an imbalance in the activity of different kinases and phosphatases (Köpke et al., 1993; Medeiros, Baglietto-Vargas and LaFerla, 2010). There are 85 known phosphorylation sites on human CNS tau, including 80 serine or threonine sites and 5 tyrosine sites(Y. Wang and Mandelkow, 2015). There are a number of antibodies used to identify pathogenic forms of tau in AD brain that mark distinct phospho-epitopes in PHF

tau. These include AT8 which marks tau phosphorylated at Ser202 and Ser205, and PHF1 that marks tau phosphorylated at Ser396 and Ser404, and monoclonal antibodies that mark phosphorylation at Ser202/205 (AT100), Thr231 (AT180), and Thr181 (AT270) (Y. Wang and Mandelkow, 2015). Hyperphosphorylated tau has less affinity for microtubules, and thus one consequence of this hyperphosphorylation is detachment of tau from the microtubules, leading to diminished microtubule stability (Hanger, Anderton and Noble, 2009). Although tau is not the only protein needed for stabilizing microtubules, as evidenced by the general health of tau knockout mice (Dawson et al., 2001; Roberson et al., 2007), loss of tau binding to microtubules due to hyperphosphorylation has been implicated in a number of biological consequences, including dendritic spine loss (Hoover et al., 2010), breakdown of the axon initial segment barrier (X. Li et al., 2011), and in axonal transport deficits (Takei et al., 2000; Vossel et al., 2010).

In addition to its canonical role in microtubule stabilization in the axon, there is increasing evidence to support a role for tau in the somatodendritic compartment as a signaling molecule and molecular scaffold. For example, it has been shown that in the presence of the Aβ, tau becomes mislocalized to dendrites, where it can bind the protein kinase Fyn and recruit it to the NMDA receptor. This tau mislocalization is thought to occur due to hyperphosphorylation of tau and its dissociation from microtubules, but is still an area of active research. This tau phosphorylation also allows for binding to PSD-95, modulating calcium influx through NMDAR and making neurons more vulnerable to excitotoxic insults (Ittner et al., 2010). It has also been shown that synaptic activation of neurons alone is enough to translocate tau to excitatory postsynaptic compartments,

suggestive of tau playing a role in synaptic plasticity and transmission (Frandemiche et al., 2014). In addition to its role at the post-synapse, tau has also been shown to facilitate signaling between the nerve growth factor and epidermal growth factor receptors, which results in increased activity from the MAP kinase pathway, and stimulation with NGF and EGF induced phosphorylation of tau at Thr231 (Leugers and G. Lee, 2010). Tau has also been implicated in playing a role in the heat shock response of cells. In one study, it was found that after heat shock of neurons, tau was recruited to the nucleus, where it was bound to nuclear DNA and facilitated DNA repair, with tau KO neurons showing significantly more DNA damage (Sultan et al., 2011).

Just as Aβ has been demonstrated to be the most toxic in its oligomeric form, recent research indicates that low-n soluble tau oligomers also cause various dysfunctions in neurons, though these species are not yet well characterized (Kopeikina, Hyman and Spires-Jones, 2012). Tau oligomers have been implicated in many deleterious effects on neurons, leading to neuron dysfunction and eventual death both in culture and *in vivo*. In cultured neurons, tau oligomers have been shown to disrupt axonal transport (Himmelstein et al., 2012; Swanson et al., 2017), and induce the translocation of endogenous tau to the synaptodendritic compartment (Swanson et al., 2017). Additionally, tau oligomers have been shown to cause impairments in cell viability and membrane integrity (Flach et al., 2012), and impair LTP in hippocampal slice cultures (Fá et al., 2016). In mouse studies, injection of tau oligomers into WT mouse brain impairs memory and induces synaptic and mitochondrial dysfunction (Lasagna-Reeves et al., 2012). In another study, injections of brain-derived tau oligomers from AD patients into hTau mice that overexpress the human form of WT tau caused tau toxicity in

neurons and cognitive impairment. However, these deficits could be rescued by treating the hTau mice with an antibody specific to the tau oligomers (Castillo-Carranza et al., 2015).

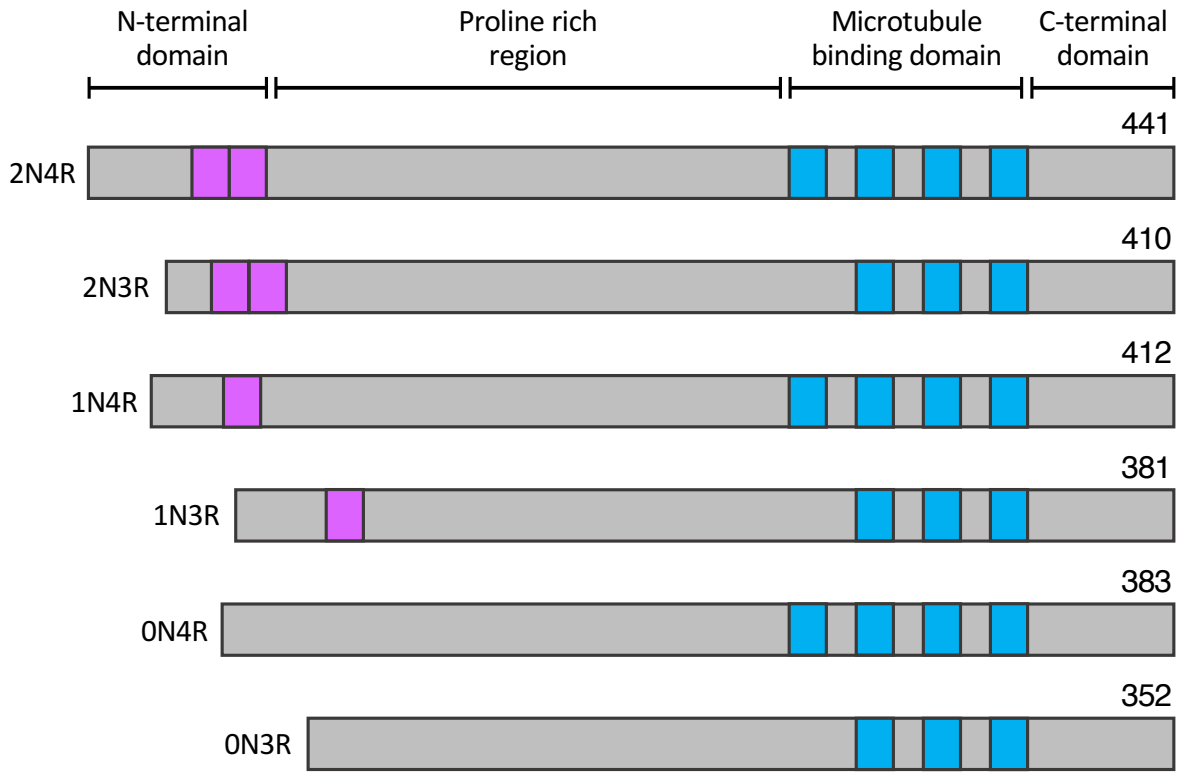


Figure 2: Structure of the six tau isoforms. Adapted from Ballatore C., Lee V., Trojanowski, J. (Ballatore, V. M. Y. Lee and Trojanowski, 2007). The tau gene, located on chromosome 17, can be alternatively spliced to create six tau isoforms. These isoforms differ in containing zero, one, or two N-terminal repeats (depicted in yellow), as well as three or four repeats contained in the MTBD (depicted in red). Thus, the six isoforms depicted here, in order from top to bottom, are known as 2N4R, 2N3R, 1N4R, 1N3R, 0N4R, 0N3R, with 2N4R being the longest isoform at 441 residues and 0N3R being the shortest at 342 residues.

8. Aβ and Tau are interconnected in AD

Although involvement of both Aβ and tau in the neuronal deficits and death seen in AD is now well established, it was not always accepted that both play important roles in the disease. For many years, it was debated whether Aβ or tau was the more causative agent of Alzheimer's disease, with those attributing AD pathogenesis to Aβ deposition being known as 'βaptists' and those believing tau was the causative agent called tauists. Those who felt Aβ was the more important molecule in disease pathogenesis cited familial forms of the disease, with all of the relevant mutations occurring in APP or the presenilin-1 subunit of γ-secretase. Tauists cited aggregated forms of tau and tangles within the neurons themselves, as well as increased levels of abnormally phosphorylated tau and decreased binding of tau to microtubules (V. Lee, 2001). Although neither party believed the other's protein had no involvement in the disease, it was not thought that both proteins acted synergistically in AD pathology. In 2001, however, a set of two papers were published in *Science* showing conclusively that amyloid and tau are connected in propagating disease pathology, which is largely synonymous with the model those in the field hold true today.

The first of these studies crossed the Tg2576 AD mouse model expressing APP with the Swedish mutation to the tau pathology model JNPL3 that express P301L mutant tau, leading to the development of tangles and progressive motor disturbance. Researchers found that the Aβ pathology that occurred in the Tg2576 line was similar to the Aβ pathology found in the double mutant in morphology, deposition, and age of onset. However, NFT deposition in the double mutant, while also morphologically similar to the NFTs found in the JNPL3 mice, appeared earlier and and spread to more

regions of the brain, notably in the olfactory cortex and limbic system. NFTs were not detected in these regions the JNPL3 mice (Lewis et al., 2001). The fact that tau pathology was accelerated in the double mutant, but amyloid pathology was not changed by the presence of mutant tau, indicates that Aβ works upstream of tangle formation, thus connecting the two pathologies.

In another study using P301L mutant tau mice, Aβ was injected into P301L mouse brain, to see whether tau pathology was accelerated. In this study, researchers prepared synthetic $A\beta_{1-42}$ fibrils, and injected these into the somatosensory cortex and hippocampus of 5-6 month old P301L mice. In addition, they had reverse peptide ($A\beta_{42-1}$ injections into P301L mice and $A\beta_{1-42}$ injections in WT mice as controls. While neither control showed an increase in tau pathology, the P301L mice injected with the $A\beta_{1-42}$ fibrils had accelerated tau pathology, showing a 5-fold increase in the number of NFTs as little as 18 days post-injection (Gotz, 2001). Similar to the other study, this research suggests that both Aβ pathology comes before tau pathology, and that Aβ induces much of the tau pathology occurring in AD brain.

Since these studies have been published, there have been numerous studies connecting amyloid and tau pathology, both in mouse models and in cultured neurons (Bloom, 2014). In addition to the studies above showing that Aβ can accelerate tau pathology, it has also been shown that reducing endogenous levels of tau in an AD mouse model ameliorates their pathology. In this study, the hAPPJ20 AD mouse model, which overexpresses APP with the Swedish and Indiana mutations, was crossed to a tau KO mouse line, and mice heterozygous and completely knocked out for tau were studied. The APP/TKO mice produced similar amyloid pathology and plaque load to the hAPPJ20

strain on its own, however, learning and memory deficits, as well as their sensitivity to excitotoxic insults, were prevented (Roberson et al., 2007). This implies a critical role for tau in mediating the pathology of AD. In a similar study, tau was knocked out in a different AD mouse model expressing mutations in APP and PS1. Here, it was found that in addition to rescuing learning and memory deficits, reducing tau also prevented synapse and neuron loss, as well as premature death of the mice seen in the original AD mouse model(Leroy et al., 2012).

In addition to these various models of APP and tau mutations in mice, there are many studies that show AβOs induce hyperphosphorylation of tau, and missorting of tau into the somatodendritic compartment (Zempel et al., 2010). One consequence of this tau missorting is tau binding to Fyn, and the recruitment of this tau-Fyn complex to the synapse, where it is able to phosphorylate the NMDAR subunit NR2B. This phosphorylation event, in turn, allows for increased calcium influx into the neuron, which mediates the excitotoxic effects of AβOs in a feed-forward loop (Ittner et al., 2010).

There is also *in vitro* data from cultured neurons showing a relationship between Aβ and tau in AD pathology. For example, the findings of the tau KO mouse study can be recapitulated in primary neurons treated exogenously with AβOs. In one study, both WT and TKO hippocampal neurons were treated with oligomerized Aβ1-40 for 24-96 hours. During this time, neurite degeneration is apparent in WT neurons, but prevented in the TKOs. Additionally, when a human WT form of tau was re-expressed in the TKO neurons, neurite degeneration was rescued, pointing to a direct role of tau in this process (Rapoport et al., 2002). Additional studies of cultured neurons have shown that tau is necessary for numerous Aβ-mediated deficits, including impairment of LTP (Shipton et

al., 2011), inhibition of mitochondria transport on microtubules (Vossel et al., 2010), and microtubule severing leading to synaptic dysfunction (Zempel et al., 2013).

Previous work from our lab has demonstrated two tau-dependent, Aβ mediated deficits. The first comes from a 2006 study, where it was shown that treatment of WT neurons with pre-fibrillar $A\beta_{1\text{-}42}$ caused microtubule disassembly. The tau dependence of this paradigm was demonstrated in CV-1 cells, which do not express tau endogenously, but were transfected with tau-GFP and YFP-tubulin constructs. When cells transfected with tau were treated with $A\beta_{1\text{-}42}$, the microtubules disassembled within 2 hours of treatment, but CV-1 cells without tau present stayed in tact upon AβO treatment (King et al., 2006). More recently, our lab has studied abberant cell cycle re-entry (CCR) in neurons, and has shown that it is an AβO-mediated, tau dependent process as well: when WT and TKO neurons are treated with AβOs, only WT neurons enter the cell cycle (Seward et al., 2013). Furthermore, inhibiting the three kinases Fyn, PKA, and CaMKII, or their subsequent phosphorylation sites on tau, inhibits CCR, demonstrating a necessity specifically for phospho-tau in initiating CCR.

9. Calcium dysfunction in AD

The divalent cation calcium is universal in terms of how its signaling regulates various processes throughout the body. Calcium's involvement in signaling ranges from microseconds when facilitating synaptic transmission, to more complicated processes such as transcription and cell division on the scale of hours to days (Berridge, Bootman and Roderick, 2003). Because calcium is such a ubiquitous signaling ion, where and when calcium is released is extremely important in determining the physiological

consequences on the cell. Typically, calcium signaling begins with a pulse of calcium, and these pulses can be very localized spanning the area of a single receptor, to large, sweeping calcium pulses that occur more globally over the surface or volume of the entire cell (Berridge, Lipp and Bootman, 2000). This calcium is typically bound to carriers or buffers within the cell, or to the effector proteins, such as calmodulin, that allow calcium to carry out various functions in the cell, with very low levels occurring freely when a cell is at rest.

While calcium is universal in terms of its signaling potential, it is an especially important signaling molecule in regulating the physiological functions of neurons. At the axon, calcium influx is necessary to trigger the release of neurotransmitter into the synaptic cleft, allowing for the propagation of signals throughout a network. At the synapse, calcium influx is necessary more as the signaling component itself, allowing calcium influx via the cell surface, through voltage-gated, receptor-mediated, or metabotropic receptors (Berridge, Bootman and Roderick, 2003). The key to these calcium bursts, that originate from extracellular calcium sources and initiate signaling paradigms, lies in the robust electrochemical gradient maintained between the intracellular and extracellular space. The resting level of calcium in a neuron is ~100 nM, whereas in the extracellular space calcium is found at ~1 mM (LaFerla, 2002). Much of the signaling that occurs at the synapse will depend on the types of receptors activated, as well as their location on the membrane. For instance, NMDA receptors on the synapse generally propagate action potentials and normal synaptic functions, whereas activation of extrasynaptic NMDA receptors can trigger excess calcium influx and signal for the death of a neuron (Hardingham and Bading, 2010). Calcium influx at the synapse is

involved mostly in synaptic transmission and regulation, whereas calcium entry at the nucleus is involved in transcriptional pathways (Berridge, 2006).

Maintaining the distinct calcium gradient between the inside of the neuron and the outside space takes a great deal of energy, and is highly regulated by intracellular stores of calcium constantly balancing the difference between levels inside and outside of the neuron. These intracellular sources of calcium are used to balance levels of calcium within the cell, but they also contribute to more long-term calcium signaling paradigms that require more nuanced regulation of calcium over time (Berridge, Bootman and Roderick, 2003). The endoplasmic reticulum, a major source of intracellular calcium, includes inositol triphosphate (IP_3) and ryanodine receptors, which release calcium in response to various upstream signaling events (Verkhratsky, 2005). IP_3 receptors release calcium in response to the second messenger IP_3, which is generated by activation of G-protein coupled receptors at the cell surface that initate a phosphoinositide signaling cascade. Ryanodine receptors are activated by increased calcium levels in the cytoplasmic compartment, in turn releasing more calcium, in a process termed calcium-induced calcium release (CICR). In addition, there are calcium exchangers, such as sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, that sequester calcium into when levels become too high in the cytoplasmic compartment (Stutzmann, 2005).

As calcium is such a fundamental regulator of neuronal function, it follows that disruptions in this network have devastating consequences. These disturbances can range from aberrant calcium influx disrupting regular synaptic function and signaling, to extreme excitotoxic shock where calcium floods the neuron, leading to oxidative stress and synaptic dysfunction leading to the eventual death of neurons. It has been

demonstrated that neurons in AD show similar signs of calcium disruption and excitotoxic stress, largely brought on by the exposure toAβOs. This is one of the main tenets of the calcium hypothesis of AD, which states that calcium dysregulation in the neuron is a necessary precursor for the etiology of AD (Khachaturian, 1985). Since the development of this hypothesis, there have been a plethora of supporting studies, with many aspects of AD pathology involving calcium homeostasis and signaling (Workgroup1, 2017).

AβOs specifically have been shown to cause numerous dysfunctions in calcium signaling. These can be divided into problems occurring at the plasma membrane via ligand-gated channels and synapses, or in the loss of regulation of intracellular calcium stores. A number of synaptic deficits in AD involve the NMDA receptor. NMDAR is a ligand-gated calcium channel that under physiological conditions binds the neurotransmitter glutamate, making the channel permeable to calcium during synaptic transmission (Cull-Candy, Brickley and Farrant, 2001). This receptor is composed of four protein subunits: two constitutive NR1 subunits common to all NMDARs, and two NR2 subunits, which have four isoforms: NR2A, NR2B, NR2C, and NR2D. The NR2 subunits contain long cytoplasmic tails, and these can influence the binding of other synaptic proteins, and can also modulate calcium influx through the channel. An NR3 subunit type exists in addition to the NR2 subunit type, though this type is less common and less well studied. In addition to its glutamate binding site, the NMDAR is gated by a magnesium ion that sits within the channel pore (Paoletti, Bellone and Zhou, 2013). Upon binding glutamate, calcium influx is contingent upon depolarization of the neuron, resulting in a

displacement of this magnesium ion, thereby allowing calcium influx through the receptor.

At the neuron surface, AβOs have been shown to allow aberrant calcium influx into neurons within microseconds of exposure; a large portion of this external calcium comes through NMDA receptors (De Felice et al., 2007; Alberdi et al., 2010; Zempel et al., 2010). This Aβ-mediated NMDAR calcium influx causes a number of problems, including impairments in LTP/LTD and learning and memory (Shankar et al., 2008), loss of synapses (Shankar et al., 2007), and increased susceptibility to excitotoxic insults (Koh, L. L. Yang and Cotman, 1990; Mattson et al., 1992). *In vivo*, when WT animals are injected specifically with AβOs (without monomers or fibrils present), similar deficits in LTP occur (Walsh, 2002). AβOs also have been shown to induce calcium influx via voltage-gated channels (MacManus et al., 2000; Rovira, Arbez and Mariani, 2002).

While most of the physiological signaling through NMDAR occurs at the synaptic junction, there are also NMDARs outside of dendritic spines, that are termed extrasynaptic. NR2B-containing NMDA receptors are generally found within the extrasynaptic regions, while NR2A-containing receptors are found predominantly at dendritic spines (Cull-Candy, Brickley and Farrant, 2001). In AD, it seems that many of the deficits at the synapse involve signaling through NR2B, and that signaling through this receptor leads to synaptic dysfunction and cell death, whereas NR2A signaling is neuroprotective (Liu et al., 2007; Hardingham and Bading, 2010). Many Aβ-induced deficits at the synapse have been attributed to NR2B-containing NMDARs, including LTP deficits (S. Li et al., 2011), spine loss and neurodegeneration (Tackenberg et al., 2013).

In addition to problems that are a direct cause of calcium signaling impairment, there are indirect problems that stem from calcium dysregulation, including disruptions in axonal transport (Decker et al., 2010), aberrant phosphorylation of tau (Mondragón-Rodríguez et al., 2012; Mairet-Coello et al., 2013) and missorting of tau into dendrites (Zempel et al., 2010; Ittner et al., 2010). Additionally, there is evidence that calcium disruptions can influence problems in metabolic signaling: when cultured neurons are treated with the drug, memantine, which blocks calcium influx through NMDAR, AβO-mediated disruptions in insulin signaling are ameliorated (W.-Q. Zhao et al., 2008).

Of note, the Alzheimer's drug memantine, an FDA approved drug that blocks excess calcium influx through NMDAR, ameliorates many of the calcium disruptions caused by AβOs, including synapse loss (Lacor et al., 2007), tau phosphorylation (L. Li et al., 2004; M. S. Song et al., 2008), and excitotoxic death in both cultured neurons (Alberdi et al., 2010) and in mice injected with AβOs (Miguel-Hidalgo et al., 2002). Additionally, memantine has shown to prevent cognitive decline and learning and memory deficits seen in AD mouse models (Minkeviciene, 2004; Martinez-Coria et al., 2010). Even within changes that occur due to aberrant calcium influx, pathology in the neuron is complex, with many of these individual problems compounding one another, contributing to the eventual death of the neuron in AD.

There is also some evidence for intracellular calcium stores contributing to increased calcium levels in AD and increased vulnerability to excitotoxicity. First, aberrant increases in calcium influx from NMDAR can lead to subsequent aberrant CICR and recruitment of ryanodine receptors, leaving the neuron more vulnerable to excitotoxic insults and altered synaptic transmission and plasticity (Chakroborty et al., 2009;

Goussakov, Miller and Stutzmann, 2010). Of important note in these studies, however, is that excess calcium occurring in this paradigm seems to be caused by presenilin mutations common in the familial form of the disease (LaFerla, 2002; K.-H. Cheung et al., 2008; Chakroborty et al., 2009). It is unclear how intracellular calcium influx caused by familial presenilin mutations would apply to the sporadic form of the disease plaguing ~99% of AD patients. Excess intracellular calcium can also contribute to other symptoms in AD, such as the production of oxidative stress and disruptions in metabolic signaling in the neuron. This, in turn, disrupts the functioning of ATP-dependent channels, such as SERCA pumps, from pumping excess calcium back into the ER and mitochondrial stores (Novelli et al., 1988; Mark et al., 1995), disrupting calcium homeostasis and making neurons more vulnerable to excitotoxic insults.

Calcium is a widely utilized signaling molecule, and because of this, when and where calcium influx occurs must be highly regulated. Even small influxes of calcium that occur erroneously can cause drastic changes to a cell's calcium balance, which in turn can disrupt the signaling pathways necessary for a neuron to function and respond to its surrounding environment. In disorders like AD, these individual disruptions to calcium compound over time, leading to neuron dysfunction and ultimately death. As preventing disruptions in calcium signaling also prevents many early pathological consequences of the disorder, however, it seems as though calcium may be a good therapeutic target for disease prevention.

10. Cell cycle re-entry in neurodegeneration

Cell division is a highly regulated and complex process, and one of the most vital to all of biology. In most biological paradigms, cells are held in check by a variety of regulators until it is necessary for them to divide. Once the cell initiates the division process, it goes through a series of events including the duplication of DNA and organelles, ultimately making its way through multiple checkpoints, and resulting in two identical daughter cells. Almost all normal differentiated neurons, however, are permanently post-mitotic, meaning that once they mature, they no longer divide. Nevertheless, it should be noted that a small amount of adult neurogenesis occurs in two regions of the brain: the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus (SGZ). This neurogenesis is associated with learning, synaptic plasticity and adolescent development, and is thought to slow down into adulthood as the brain ages (He and Crews, 2007; C. Zhao, Deng and Gage, 2008).

A phenomenon that is less understood in AD and numerous other models of neuron injury is ectopic neuronal CCR. This phenomenon was first observed in the 1990s, and since that time has been observed in human AD brain, numerous mouse models of AD, and in cultured neurons, each of which will be discussed in more detail in this section. The first evidence for aberrant CCR leading to neuron death came from studies of brain development, in mice knocked out for the tumor suppressor protein, retinoblastoma (Rb). In developing WT mouse embryos, as well as in human development, neurogenesis is reserved to the neural tube within the VZ, with these cells migrating out of this region as they complete cell division and settle into various regions, eventually becoming post-mitotic. As shown by bromodeoxyuridine (BrdU)

incorporation via injection into pregnant Rb-KOs, there are a significant number of mitotic cells throughout the CNS and PNS, in areas that are generally reserved for post-mitotic cells, indicating neurons in this region never became post-mitotic. Furthermore, it seems that the same neurons going through the cell cycle in these areas are eventually dying distinctly from apoptosis, as indicated by TUNEL-positive cells all throughout the CNS (E. Y. Lee et al., 1992; 1994). Another lab contemporaneously generated a different Rb-KO mouse, and also found that neurons in the CNS died via apoptosis (Jacks et al., 1992). Paralleling these studies were studies in which SV40 was expressed in Purkinje neurons of the cerebellum, thus driving these neurons into the G1-S phases of the cell cycle by inhibiting the suppressor function of Rb. In these mice, SV40 was expressed specifically after neuronal precursors differentiated into post-mitotic neurons, and this expression led to eventual apoptosis of these neurons, presumably by driving them back into the cell cycle after their differentiation (Feddersen et al., 1992). In a follow up study, this group found that SV40 induced cell death of these post-mitotic Purkinje neurons required SV40 to bind pRb to push neurons out of their post-mitotic state. Once 'mitotic', these neurons proceeded through the cell cycle up to M phase, as evidenced by BrdU incorporation, but died via apoptosis before completing mitosis (Feddersen et al., 1995). Another way in which neurons can be forced to re-enter the cell cycle is by knocking out Cdk5, a cyclin-dependent kinase that inhibits cell cycle activity in neurons, keeping them in a post-mitotic state. In mice that are completely knocked out for Cdk5, neurons show neuronal migration deficits, expression of proliferating cell nuclear antigen (PCNA) indicating CCR, and positive staining for caspase 3 and nicked DNA in the neocortex, indicating apoptosis has occurred (Cicero and Herrup, 2005).

Even in situations where CCR is not induced, there are instances of CCR occurring in models of neuronal death. For example, there are two different mouse lines, *staggerer* and *lurcher*, that both have substantial neuron loss in Purkinje neurons and cerebral granual cells due to lack of trophic support. Before the loss of these neurons in these mice, however, expression of the cell cycle markers cyclin D1, PCNA, and BrdU incorporation were seen (Herrup and Busser, 1995). In an excitotoxic cell death paradigm, where mice are injected with kainic acid to induce high levels of calcium influx in excitatory neurons, there is a significant increase in cyclin D1 positive neurons in the CA1 and CA3 regions of the hippocampus (Park et al., 2000). Increased levels of cyclin D1 mRNA in the hippocampus were also found in a similar study, where ischemia was induced via injection of kainic acid into the amygdala or by four vessel occlusion generating ischemia in the forebrain (Timsit et al., 1999).

In addition to what occurs in mice, several *in vitro* studies reveal other paradigms in which neuronal death is preceded by markers of the cell cycle. The first example of this came from cultured sympathetic neurons that were deprived of nerve growth factor (NGF). NGF deprivation induces cell death in neurons, but before these neurons die, they show a significant upregulation of cyclin D1 RNA (Freeman, Estus and E. M. Johnson Jr, 1994). In this same model of NGF deprivation of sympathetic neurons, death can be prevented by pretreatment of neurons with 3 different cell cycle inhibitors, mimosine, ciclopirox, and deferoxamine, that specifically inhibit the G1 to S transition of the cell cycle. Interestingly, inhibitors that block the S-, G2-, and M- phases of the cell cycle did not rescue neuron death in this paradigm, supporting the idea that neurons die before they are able to complete DNA duplication. In a model similar to excitotoxicity, primary

neurons were treated with 3-nitroproprionic (3-NP) to induce oxidative stress via inhibition of the electron transport chain, resulting in neuron death. Interestingly, when these neurons were treated with the cyclin dependent kinase (CDK) inhibitor flavopiridol, neurons were partially protected from this death (Park et al., 2000).

CCR is also thought to contribute to much of the cell death occurring in AD, as evidenced from human AD patients, AD mouse models, and models of AD in culture. Some of the first evidence for the involvement of CCR machinery in the pathogenesis of AD came from phospho-epitopes detected on tau found in the tangles of AD patients. When these phospho-tau epitopes were expressed in dividing cell lines, it was found that the highest level of phosphorylation was detected when cells were in M-phase of the cell cycle (Vincent, Rosado and Davies, 1996). The authors postulate based on this observation that mitotic kinases are responsible for some of the phospho-epitopes seen in the tangles of AD patients. In a follow up to this study, the authors found that Cdk1 is upregulated in AD brains, and that specifically they see Cdk1 upregulation near NFT-positive neurons that also have an increase in cyclin B1 levels (Vincent et al., 1997).

There are also studies showing the upregulated expression of numerous cell cycle proteins in AD brain. In one study, the cell cycle markers cyclin D1, cyclin B1, cdk4, and PCNA were shown to be upregulated in the hippocampus, subiculum, locus coeruleus, and dorsal raphe nuclei of AD patients, but not in areas that are relatively unaffected in AD such as the inferotemporal cortex and cerebellum (Busser, Geldmacher and Herrup, 1998). Another study from around the same time found increased levels of cyclin B1 in AD brains in the dentate gyrus, subiculum, and CA1 regions of the brain, but nowhere in healthy patient controls (Nagy et al., 1997). This group also found increases in Ki67, a

protein present in all phases of the cell cycle but not in G0, was upregulated in the hippocampus of AD patients, with the highest expression in the dentate gyrus (Nagy, Esiri and Smith, 1997). Similarly, another group reported increases in Cdk4 and its inhibitor, p16, in the pyramidal neurons of the hippocampus in AD patients, and speculate that p16, which is normally not expressed in mature neurons, is a response to increased levels of Cdk4 to counteract these neurons going into the cell cycle (McShea et al., 1997).

Evidence that CCR is an early pathologic event in the course of AD progression comes from the study of mild cognitive impairment (MCI) patients, many of whom would have eventually progressed into AD: in these cases, expression of PCNA and cyclins B and D was significantly increased in the entorhinal cortex and the hippocampus, with the MCI samples showing higher instance of CCR than samples of patients that had already progressed into AD (Y. Yang, Mufson and Herrup, 2003). This study is of particular interest, as it gives insight into the time frame of CCR: it seems as though this phenomenon occurs at the earliest stages of disease progression, and continues throughout the course of AD. In all of these instances, there is no evidence that these neurons are completing cell division: rather, it seems that these neurons make it partially through S phase before succumbing to death.

In addition to staining for protein markers of neuronal CCR, there is evidence that large portions of chromosomes become replicated in neurons before they die. Evidence for this comes from in-situ hybridization in areas of the brain showing pathology in AD. In healthy tissue, normal diploid chromosomal complement was found in all neurons, however, in AD patient samples, there was aneuploidy in hippocampal pyramidal and

basal forebrain neurons in at least four different loci on 3 separate chromosomes (Y. Yang, Geldmacher and Herrup, 2001). This indicates that not only are CCR proteins being expressed in neurons, but that these cells are going through the cell cycle at least partially through S-phase. In line with this is a study of hyperploid neurons in various states of AD progression. In this study, patient samples from various stages of disease progression were examined for hyperploidy via *in situ* hybridization, and the entire parahippocampal gyrus was scanned for total DNA content and a probe to a locus on chromosome 17, for a total of 80,000-120,000 neurons counted per sample. Patients were analyzed for hyperploid content and then aligned based on disease progression. What the researchers found was that as the disease progressed initially, the number of hyperploid neurons increased, but as patients progressed from mild- to severe AD, the number of hyperploid neurons decreased. Of important note here is that although a significant number of hyperploid neurons were lost over the course of AD, the number of diploid neurons remained unchanged regardless of disease progression, supporting the hypothesis that hyperploid neurons are the ones susceptible to death in AD (Arendt et al., 2010).

Additional evidence for neuronal CCR playing a role in the pathology of AD comes from AD mouse models and culture models of AD. Numerous mouse lines created to mimic the pathology of AD show signs of CCR. In one study of various AD mouse models, all expressing mutant forms of APP and/or PS1 and showing Aβ plaque pathology were all positive for the CCR markers PCNA and cyclin A in the cortex and hippocampus (Y. Yang et al., 2006). Notably, the expression of these CCR markers came at least 6 months before significant plaque pathology occurred in each of the mouse lines, suggesting that CCR is a precursor to other, more canonical pathological markers, such as

plaques and tangles. In a follow up study, the R1.40 mouse line overexpressing APP_{SWE} was studied more extensively. Here, it was shown that the R1.40 mouse line starts showing the CCR markers PCNA and cyclins A and D, and aneuploidy as shown by FISH at 6 months of age, 7 months before the onset of plaques in the brain. This result is followed up by crossing the R1.40 mouse to a BACE1 knockout, precluding the formation of β-CTE and Aβ, and finding that this inhibits CCR. This indicates that Aβ, in some form, is necessary for inducing neuronal CCR. The importance of Aβ in inducing CCR has been confirmed in culture by treating primary neurons with either monomeric or oligomeric Aβ. It was observed that AβOs specifically cause these neurons to re-enter the cell cycle, and treatment with monomeric Aβ or vehicle control does not induce CCR. This supports the idea discussed in the Aβ section of this thesis, that oligomeric Aβ is the toxic species of amyloid in the brain. This result has been followed up in our own lab by showing that AβOs, and not monomeric or fibril Aβ, induce CCR, and that induction of CCR is dependent on the presence of tau (Seward et al., 2013). Furthermore, this pathway is dependent on AβO-mediated activation of mTORC1 at the cell surface and subsequent phosphorylation of tau (Norambuena et al., 2017), and is also dependent on calcium signaling through NMDAR. Finally, it has been demonstrated by the primary results of this dissertation in Chapter 2.

What is still unclear in these hypotheses is how, exactly, these neurons are dying, and the rate at which these neurons are dying. In some of the instances above, TUNEL and cell morphology analysis point to an apoptotic cell death. The process of neuron death in these instances seems to be an active process rather than dying by necrosis. The expression of different CCR related proteins related to death is also in line with the

hypothesis that death in these instances is programmed. Complicating this matter further is the fact that the signs of CCR seem to be present for longer than a canonical cell death program would take to clear the neuron and thus any sign of CCR. One hypothesis put forward, though still not conclusive, is the 'two-hit' hypothesis of AD (X. Zhu et al., 2004). This postulates that CCR or other single dysfunctions alone, such as oxidative stress, are not enough to signal for cell death in the neuron, but rather that it takes multiple insults occurring in the neuron for it to succumb to death. This makes sense in terms of the time frame of AD itself—patients can live with this disease for years or even decades before death. This can be partially explained by the plasticity of the brain, and an individual's cognitive reserve. However, it is clear that cell death is occurring over a much longer time frame than average cell death. Another theory as to why CCR occurs at all was proposed by Thomas Arendt and Martina Bruckner. It has been shown that some cell cycle proteins are repurposed in neurons to control synaptic plasticity. What the authors propose is that when a neuron is stressed or sick, this machinery can be repurposed aberrantly, driving neurons into the cell cycle to try, futilely, to divide (Arendt and Brückner, 2007). Support for this theory also comes from the fact that it seems holding the cell cycle in place is an active process. Rather than the neuron becoming post-mitotic by losing the ability to divide, it seems that neurons are always being held in check by cell cycle inhibitors. Part of the dysregulation in neurons, it seems, is the loss of this hold on cell cycle re-entry, and thus neurons attempt fruitlessly to divide (Herrup and Y. Yang, 2007a).

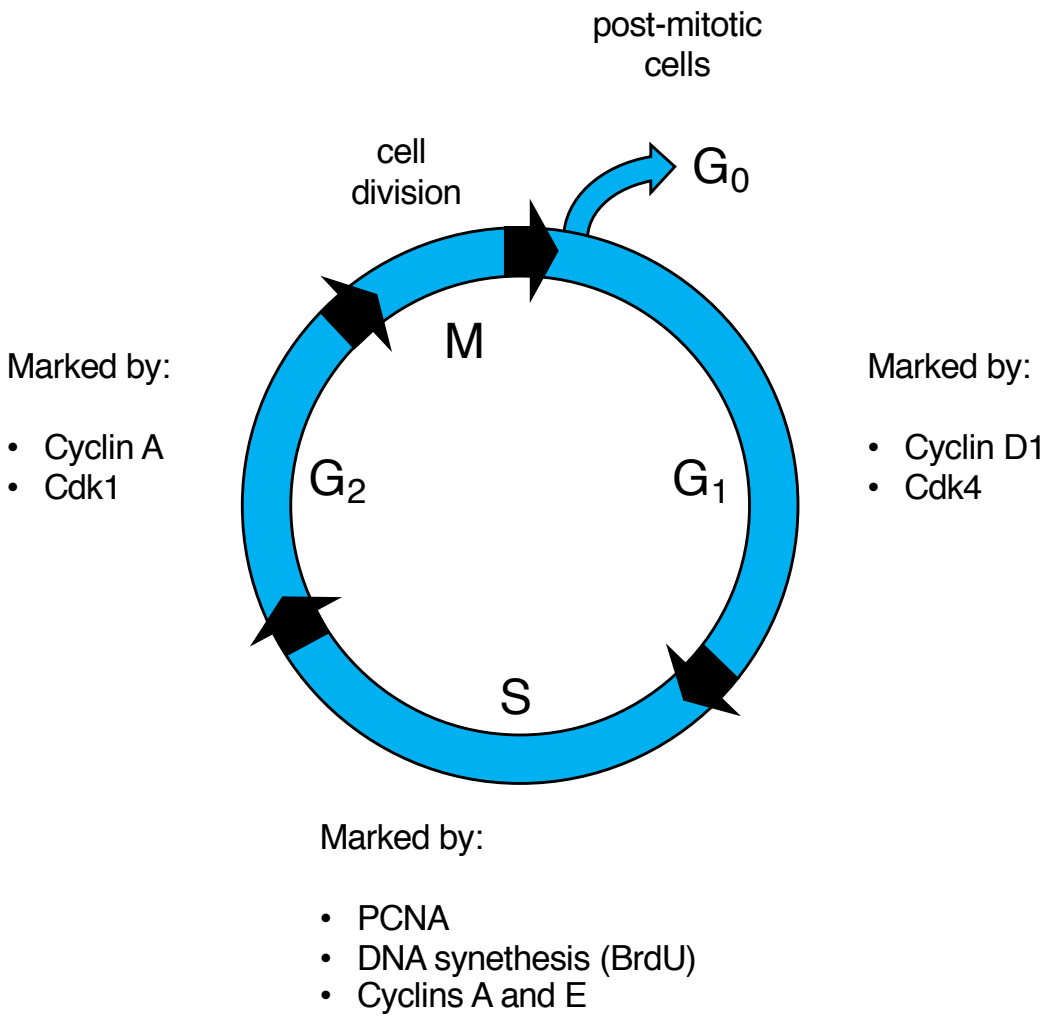


Figure 3: Schematic of neuronal CCR and staining of various markers in human AD brain. Adapted from Arendt, T., 2012. Staining of human AD brain sections depicts numerous markers of CCR at various stages of the cell cycle. Rather than the neuron proceeding to M phase or attempts to divide, however, it appears that these CCR positive neurons die instead.

Chapter 2

The cell cycle re-entry pathway of neuron dysfunction in Alzheimer's disease shares a mechanistic origin with amyloid-β oligomer-induced excitotoxicity

The following chapter has been adapted and expanded from a publication by Kodis, et. al.

1. Introduction

Alzheimer's disease (AD) is a devastating neurological disorder characterized by memory loss and cognitive decline. These behavioral symptoms are caused at the cellular level by synaptic dysfunction and loss, and neuron death, and at molecular level by toxic forms of amyloid-β (Aβ) and tau that work coordinately to damage synapses (Selkoe, 2002; Ittner et al., 2010; Sheng, Sabatini and Südhof, 2012) reduce insulin signaling (la Monte, 2014), impair axonal transport (Vossel, 2010), and kill neurons (Rapoport et al., 2002; Nussbaum et al., 2012; Bloom, 2014). While poorly soluble, fibrillar forms of Aβ and tau that respectively are found in plaques and tangles are histopathological hallmarks of AD, soluble oligomeric forms of Aβ and tau are now widely recognized as being far more toxic (Walsh et al., 2002; Hardy and Selkoe, 2002; Lacor et al., 2007). It follows naturally that efforts to prevent and treat AD will benefit from advances in our still primitive understanding of the pathogenic signaling mechanisms that underlie the breakdown of normal neuronal homeostasis caused by toxic oligomers of Aβ and tau.

As much as 90% of neuron death in AD may follow ectopic re-entry of neurons into the cell cycle (Herrup and Y. Yang, 2007b; Arendt et al., 2010). Whereas fully differentiated, healthy neurons are permanently post-mitotic, affected neurons in AD and other neurodegenerative disorders often express molecular markers of the G1 and S-phase stages of the cell cycle

(Y. Yang, Mufson and Herrup, 2003; Greene et al., 2007; Varvel et al., 2008; Arendt, 2012). Instead of dividing, however, these neurons apparently die after a delay of up to hundreds of days following cell cycle re-entry (CCR) (Y. Yang, Geldmacher and

Herrup, 2001). Previous research from our laboratory defined a CCR signaling network in AD, whereby Aβ oligomers (AβOs) induce activation of multiple protein kinases that catalyze site-specific tau phosphorylation, and a positive feedback loop between phospho-tau and the multi-subunit protein kinase complex, mTORC1, that leads to mTORC1 dysregulation and drives neurons from G0 into G1 (Seward et al., 2013; Norambuena et al., 2017).

Besides triggering CCR and neuron death, AβOs also cause excitotoxicity by stimulating excess calcium influx into neurons (Khachaturian, 1989; Mattson et al., 1992; Workgroup1, 2017). This disruption of normal calcium homeostasis affects numerous signaling pathways, and can damage and destroy synapses, and lead to abrupt neuron death (Y. Wang and Qin, 2010). A major contributor to this pathological process is the N-methyl-D-aspartate receptor (NMDAR), which permits toxic levels of calcium to enter neurons exposed to AβOs (Ittner et al., 2010; Malinow, 2011). Interestingly, one of the few FDA-approved treatments for AD is memantine, which works by blocking excess calcium entry into neurons via the NMDAR (Lipton, 2006).

The study described here tested the hypothesis that neuronal CCR and excitotoxic calcium influx via NMDAR share a common mechanistic origin initiated by AβOs. Using AβO-treated primary mouse neuron cultures, we show that CCR is prevented by chelating total cellular calcium, by pharmacologically blocking AβO-mediated calcium influx through NMDAR, or by reducing expression of an NMDAR subunit protein. Moreover, we found that neuronal CCR *in vivo* in Tg2576 AD model mice can be blocked by treating the mice prophylactically with memantine. Taken together, these results mechanistically link AβO-induced calcium influx and the resultant excitotoxicity

and neuronal CCR. Moreover, they suggest that memantine, which is used as a drug for temporarily and modestly relieving symptoms in patients with a clinical AD diagnosis, but does not apparently act as a disease-modifying drug in that context, has the potential to forestall disease progression if administered during pre-symptomatic stages of the disease.

2. Materials and methods

1. Animals and Usage: All animal usage and protocols were approved by the IACUC of the University of Virginia. Animals were housed in a barrier facility with ad libitum access to food and water on a 12-hour light/dark cycle.

2. Neuron dissections and cultures: Cortices from C57/BL6 WT mice at age E17-E18 were used for primary neurons cultures. Unless otherwise stated, reagents are from Gibco/Life Technologies. To make primary neuron cultures, brains were removed from pups and cortical tissue removed from each lobe after removing meninges. Cortical tissue was diced into ~ 1 mm^2 pieces and incubated in 0.25% trypsin without EDTA in Hank's balanced salt solution (HBSS) at 37 °C for 45 minutes with shaking every 5 minutes. After digestion, heat inactivated fetal bovine serum (FBS; HyClone/GE Healthcare) was added to the tissue mixture at a 1:1 ratio to quench peptidase activity, followed by 3 washes of 5 mL with HBSS. After washing, the tissue was incubated with 500 units/mL HBSS DNAse I (Worthington Biochemical) to degrade extracellular DNA, and then triturated to a single cell suspension. Cells were then counted using a hemocytometer and plated at a density of 50,000 cells/cm^2 on glass coverslips or plastic coated with 50 μM

poly-D-lysine overnight at 4 °C. Neurons were maintained in a humidified incubator with 5% CO2, with 50% media changes after day 7 and every 2-3 days after until day 16-18 when treatments occurred.

3. Amyloid beta oligomerization and treatment: Lyophilized amyloid beta 1-42 peptide purchased from Anaspec was resuspended in1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and let sit for 4 hours to let all peptide dissolve. After peptide was dissolved, 2 mM aliquots of 20 μL were made and stored at -80 °C until ready for use. To oligomerized the peptide, first aliquots were dried in a speed vac for 3-4 hours, creating a peptide film. This peptide was then solubilized to 1 mM in DMSO, spun down to ensure all peptide was in solution, and then sonicated in a water bath sonicator for 5 minutes to break up aggregates and ensure peptide was completely solubilized. After sonication, the peptide was brought up to a final concentration of 100 μM plain neurobasal media. The solution was then placed on an orbiting rocker at 4 °C for 48 hours, then spun at 14,000 x g for 15 minutes to get rid of insoluble fibrils. Neurons were treated with 3 μM AβO initial monomer concentration, corresponding to a range of 1.5-2 μM soluble species after oligomerization and centrifugation.

4. Immunofluorescence of primary neurons: All steps were performed at room temperature unless indicated otherwise. After treatment with AβOs for 16-18 hours, primary mouse cortical neurons grown on #1.5 thickness, 12 mm round glass coverslips were rinsed once with ice-cold phosphate buffered saline (PBS), and then were fixed in 4% paraformaldehyde in PBS for 15 minutes. Following fixation, the cells were rinsed 3 times for 5 minutes each with ice cold PBS, then permeabilized for 15 minutes with PBS

containing 0.25% Triton X-100 (Fisher, 9002-93-1), and rinsed 3 times for 5 minutes each with PBS. Next, the cells were blocked with 2% bovine serum albumin (BSA; Roche 03116956001) in PBS +0.1% Tween20 (PBST; Fisher, 9005-64-5) for 1 hour, after which they were labeled overnight with primary antibodies followed by secondary antibodies for 1 hour. Primary antibodies were diluted into 2% BSA in PBST to increase specificity of the antibody, and secondary antibodies were diluted in 2% BSA in PBS. After each antibody incubation step, the cells were rinsed 3 times for 5 minutes each with PBS. Finally, the coverslips were rinsed with ultrapure water and mounted onto slides using Fluormount-G (Southern Biotech, 0100-01) containing 1% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma, D27802-25MG), an anti-quenching agent.

5. Western blotting: Samples were run on a 12% SDS-PAGE gel, then transferred at 100 V for 1h 45m onto a 0.22 μm nitrocellulose membrane. After transfer was complete, blots were rinsed 1x in TBS, then blocked for 30 minutes to 1 hour in TBS blocking buffer (Licor). After blocking, membranes were incubated in primary antibody in antibody buffer +0.1% tween (1:1 dilution of Licor TBS blocking buffer and TBS). Secondary incubation was done with Licor IR secondaries in blocking buffer. Between antibody incubations membrane was rinsed 3 times for 5 minutes each in TBS (primary in TBS+0.1% tween). Then, membrane was incubated in Licor secondaries as indicated (Goat anti-rabbit 800; Goat anti-mouse 680) for 1 hour. Membrane was then dried in blotting paper, then scanned using the Licor Odyssey imaging station.

6. Virus Production: HEK cells were grown in culture to approximately 90% confluency in 15 cm^2 round dishes in DMEM, then transfected with envelop, packaging, and shRNA

plasmids as indicated using Lipofectamine 3000. In one 15 mL conical, 60 μL of Lipofectamine was added to 2 mL OptiMEM media per KD construct to be transfected (i.e., for two viral KD constructs, use 4 mL of OptiMEM and 120 μL Lipofectamine 3000 reagent). Since this mixture is the same for each viral construct to be made, the mixture can be made in one tube with the appropriate volume per viral construct (2 mL per virus to be made). In separate 15 mL conical tubes, using one tube per each viral construct, add 2 μL of the P3000 reagent (separate from the Lipofectamine itself) was added per 1 μg of DNA. The total amount of DNA used per construct can vary between 15-60 μg of DNA depending on the virus titer at the end of production. For each construct used in this work, a total of 30 μg DNA was used in the following ratios: 50% shRNA vector, 37.5% pMD2.G envelope vector, and 12.5% psPAX packaging vector. Since a total of 30 μg of DNA was used, 60 μL of the P3000 reagent was added to each viral KD construct to be transfected. Each mixture was allowed to incubate for 5 minutes separately, then 2 mL the Lipofectamine 3000 mixture was added to each DNA mixture dropwise, carefully avoiding the sides of the tube to ensure Lipofectamine micelles stay in tact. This mixture was then allowed to incubate for 20 minutes to allow for DNA incorporation. During this time, media in the 15 cm2 dishes was removed and 13 mL DMEM complete media was added back to the HEK cells such that the final volume of each dish was 17 mL once the Lipofectamine mixture was added. After DNA-Lipofectamine complexes were formed, each mixture was added dropwise to a single 15 cm2 dish, covering as much area as possible. Plates were then placed into an incubator, and media was collected at 24 and 52 hours post-transfection. At 24 hours, 17 mL of media containing virus was removed from each dish, placed into a 50 mL conical, and placed at 4 °C until ready to collect virus, and

17 mL of complete DMEM was added to the cells again until the next collection at 52 hours. Once all of the media was collected, the virus was pelleted in a Beckman Coulter Optima LE-80K ultracentrifuge for 2 hours at 23,000 rpm (95,000 x g) at 4° C in a SW28 rotor. The supernatant was then removed and the pellets containing the virus was resuspended in 300 µl Neurobasal medium. Viruses were then aliquoted and stored at -80 C until ready for use. Mission shRNAs for the knockdown vectors were obtained from Sigma-Aldrich (NR1 knockdown vector: TRCN0000233327, PSD95 knockdown vector: TRCN0000321881).

7. Memantine treatment: Tg2576 AD model mice that overexpress human β-amyloid precursor protein (APP) with the K670N/M67IL Swedish mutation (Hsiao et al., 1996) and WT mice of the same background (50% SLW, 50% C57/BL6) were treated with memantine at ~30 mg/kg/day based on a previously described protocol from the time they were weaned (3 weeks) until 2 months of age. The memantine concentration in water was ~1 mM, chosen based on mouse weight and the assumption of mice consuming 0.15 ml of water per g body weight per day. Mice were given *ad libitum* access to food and water.

8. Perfusion and tissue sectioning: After memantine treatment, mice were perfused using a ketamine/xylazine cocktail provided by the Center for Comparative Medicine at the University of Virginia and dosed according to IACUC guidelines. Mice were transcardially perfused first with 10 ml of 0.1 M phosphate buffer (PB) at pH 7.6, followed by 10 ml of 4% paraformaldehyde in PB at a flow rate of 1.5 mL/minute. Brains were then removed immediately and placed in 4% paraformaldehyde in PB for 24 hours, washed 3

times in PBS, incubated in 30% sucrose overnight until brains were saturated, and flash frozen using dry ice. Frozen tissue was stored at -80° C until ready for sectioning, then sagittal sections were cut to 40 μm thickness on a Microm HM 505 E cryostat and stored in PBS at 4 C until ready for staining.

9. Tissue section staining: Protocol adapted from Abcam's free floating section immunofluorescence protocol. Sagittal brain sections were placed into wells of a 12-well tissue culture dish, rinsed briefly with PBS, then subsequently rinsed 3 times for 15 minutes each in PBS + 0.3% Triton X-100 to permeabilize the tissue. Sections were then blocked by incubating in PBS + 0.3% triton and 2% normal goat serum for 2 hours at RT with gentle rocking. Sections were then placed in primary diluted in PBS + 0.1% Triton X-100 overnight at 4 C, rinsed, and placed in secondary diluted similarly to primary for 2 hours at room temperature. After staining, sections were washed three times in PBS, and mounted in Fluoromount-G + 1% DABCO.

10. Microscopy: For *in vitro* immunofluorescence experiments, cells were first imaged on an EVOS FL cell imaging system with a 20X 0.4 NA objective (ThermoFisher Scientific) to quantify CCR per condition. For tissue section quantification and all immunofluorescence images used in figures, cells were imaged using a Nikon Eclipse Ti equipped with a Yokogawa CSU-X1 spinning disk head with the 40X 1.3 NA CFI S Fluor objective, using the 405 nm, 488 nm, 561 nm and 640 nm lasers, and processed using the Nikon Elements software and Adobe Photoshop. For immunofluorescence, n=4 biological replicates with >300 neurons counted per condition per experiment. For

memantine sections, four brains from each experimental condition were counted, and for each brain, 3 sections were imaged, and for each section 3 images or > 1,500 neurons were counted to quantify cyclin D1 positive neurons.

11. Statistics: All statistical analysis was performed with Prism 7 software. For BAPTA and MK-801 CCR experiments, one-way ANOVAs were used for analysis. For pCaMKII time courses with MK-801 and shRNA, two-way ANOVAs with Bonferroni post-hoc test to give p values for individual time points. For Tg2576 memantine experiments, one-way ANOVA was used. For each section imaged, neurons from at least 3 frames were counted, and for each mouse, 3 total sections were imaged: one lateral, one medial, and one in the middle. At least 3,000 neurons were counted per mouse to get CCR totals for each condition.

12. Other reagents used:

Drugs: BAPTA-AM (ThermoFisher; B1205), MK-801 (Sigma; M107-5MG), memantine (Sigma; M9292-100MG).

Primary Antibodies: chicken anti-MAP2 (abcam; ab92434), mouse anti-NeuN (Millipore; MAB377), rabbit anti-CyclinD1 (abcam; ab16663), rabbit anti-NR1 (CST;D65B7), rabbit anti-PSD95 (CST; D27E11), rabbit anti-pCaMKII (CST; D21E4), mouse anti-total CaMKII (BD Labs; 611292), mouse anti-βIII-tubulin; (TuJ1; courtesy of T. Spano, University of Virginia),

Secondary antibodies: goat anti-mouse IgG Alexa Fluor 568 (LifeTechnologies; A11041), goat anti-mouse IgG Alexa Fluor 405 (Invitrogen; 35501BID), goat anti-rabbit IgG Alexa Fluor 488 (LifeTechnologies; A11034), goat anti-chicken IgG Alexa Fluor 647

(LifeTechnologies; A21235), goat anti-mouse IRDye 680LT (Licor; 925-68070), goat anti-rabbit IRDye 800 CW (Licor; 925-32211.

Results

1. Intracellular Calcium is Necessary for CCR

To test the hypothesis that neuronal CCR, defined in this context as the presence of the G_1 marker cyclin D1, is calcium-dependent we first treated primary mouse cortical neurons with BAPTA-AM, a cell-permeant chelator of intracellular calcium, beginning 30 minutes prior to addition of AβOs to the cultures. After 16-18 hours of AβO exposure, neurons were fixed and stained by triple immunofluorescence microscopy with antibodies to the G1 marker, cyclin D1, the neuron-specific nuclear protein, NeuN, and the neuronal somatodendritic protein, MAP2. As shown in Fig. 1, AβO treatment increased the fraction of cyclin D1-positive neurons from ~8% to ~25%, but the rise in cyclin D1-positive neurons was prevented by pre-treatment with BAPTA-AM. These results demonstrate that calcium is necessary for neuronal CCR.

Figure 1:

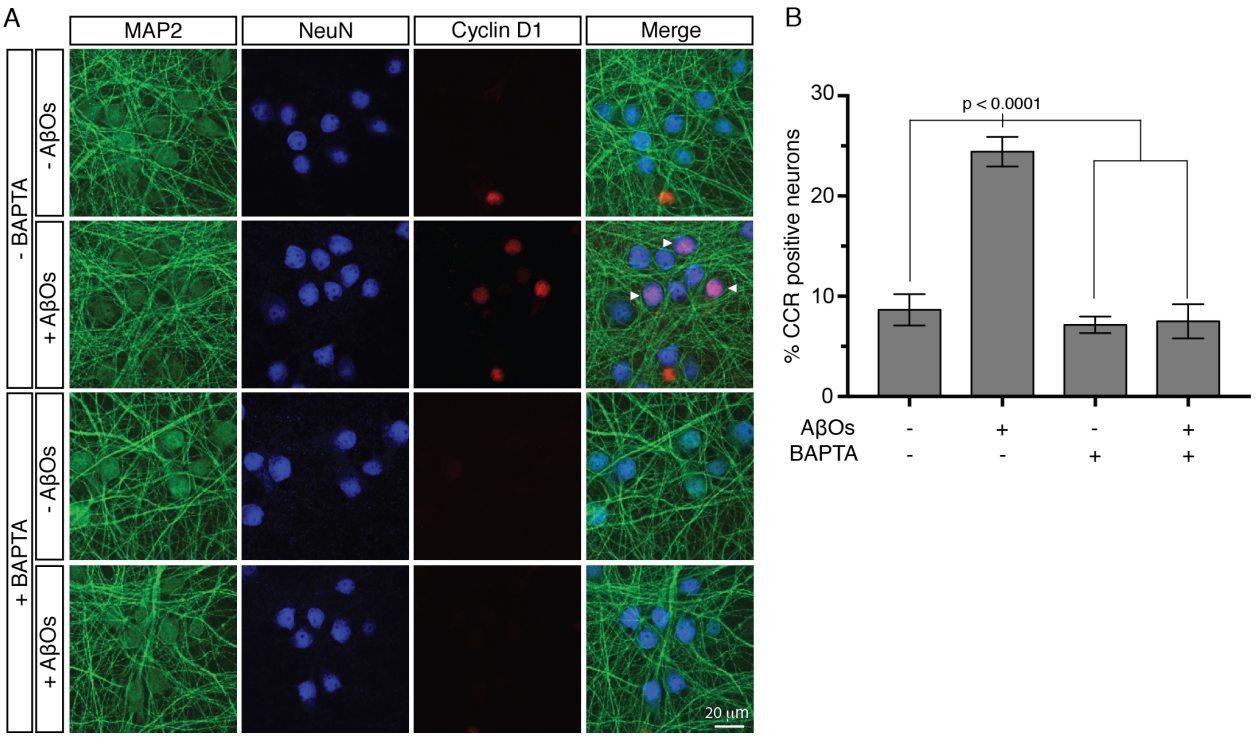


Figure 1: Intracellular calcium chelation by BAPTA-AM prevents AβO-induced neuronal CCR. (A) Primary cortical neurons were treated for 18 hours with AβOs with or without 10 μM BAPTA-AM to chelate intracellular calcium. CCR neurons were identified by their immunoreactivity with antibodies to both cyclin D1, which marks nuclei during G1 of the cell cycle, and NeuN, which marks nuclei in all neurons. Neuronal somatodendritic compartments were labeled with an antibody to MAP2. (B) Quantification of the immunofluorescence results. Results have $p < 0.0001$ by one-way ANOVA.

2. Blocking the NMDA receptor pharmacologically with MK-801 blocks CCR and prevents Aβ*O-mediated early activation of CaMKII via NMDAR*

We next investigated which specific calcium sources contribute to the initiation of CCR. Given the importance of NMDAR in synaptotoxicity and AD, we tested whether calcium influx through this receptor is required for CCR. Accordingly, primary neuron cultures were treated with MK-801, an NMDAR inhibitor that blocks calcium entry through the channel pore, beginning 30 minutes before AβOs were added. After 16-18 hours of AβO treatment, neurons were fixed and stained for NeuN, MAP2 and cyclin D1 to enable quantitation of neuronal CCR. As shown in Fig. 2A, pre-treatment of neurons with MK-801 blocked the induction of CCR by AβOs. We therefore conclude that NMDAR is the major, and possibly exclusive source of the excess calcium that enters neurons in response to AβO exposure and initiates CCR.

Previous work from from our lab (Seward et al., 2013) demonstrated that AβO-mediated CCR is contingent on activation of at least three protein kinases: CaMKII, the src-family kinase, Fyn, and protein kinase A (PKA). CaMKII is of particular interest, as activation of this kinase occurs via calcium influx through NMDAR. This led us to test the hypothesis that AβO-mediated calcium influx through NMDAR activates CaMKII as a mediator of NMDAR's role in CCR. Again, we treated primary neurons with AβOs with or without 30 minute pre-treatment with MK-801, and collected the cells at various time points between 15 minutes and 2 hours after AβO stimulation for western blotting analysis with antibodies to phospho-activated and total CaMKII. As shown in Fig. 2C, we found that CaMKII was transiently activated 15 minutes after AβO addition in the absence of MK-801, and that activation was prevented by MK-801. The AβO-mediated activation of

CaMKII necessary for CCR (Seward et al., 2013) is therefore dependent upon calcium influx through NMDAR.

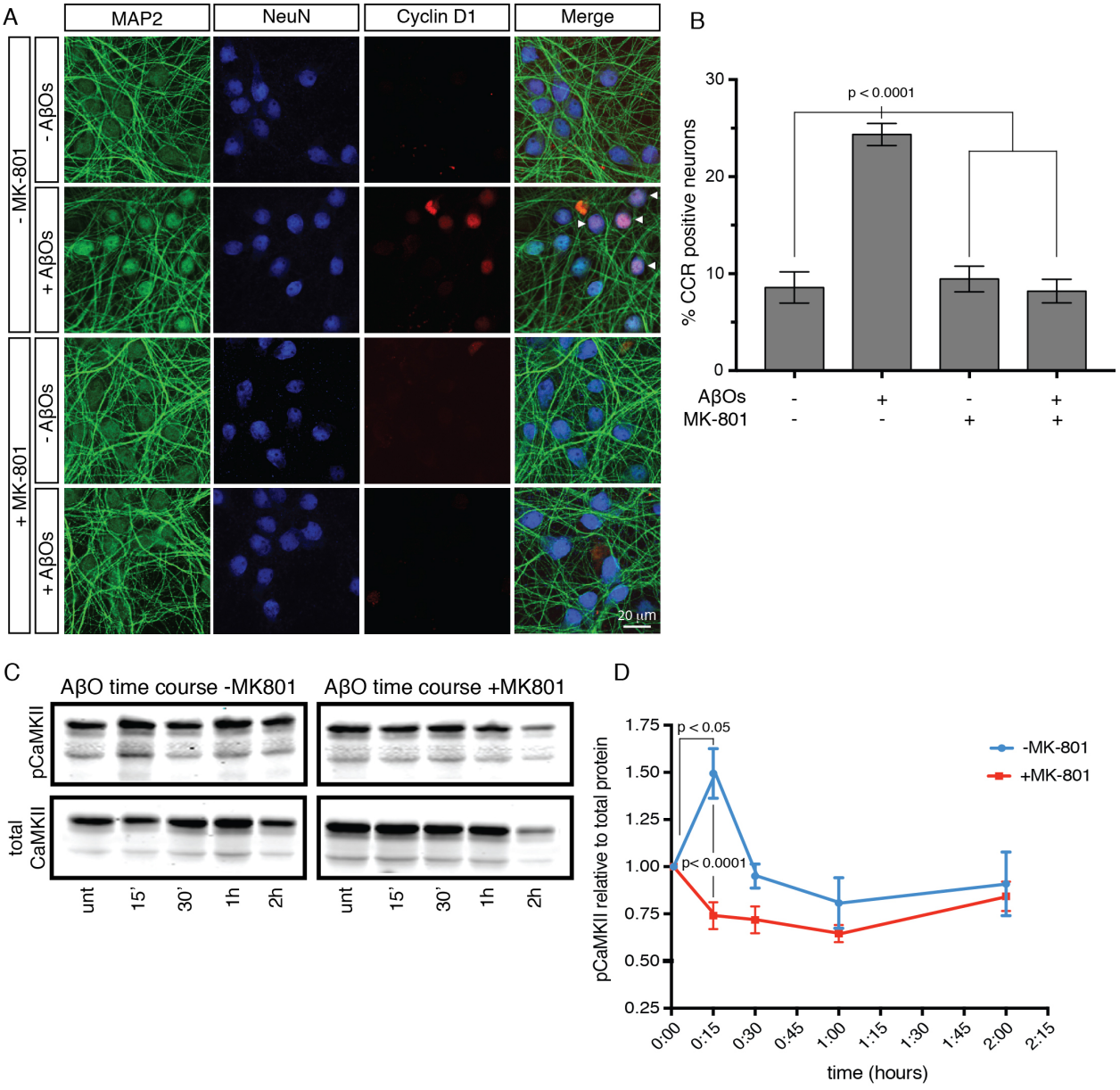

A
MAP2
NeuN
Cyclin D1
Merge
- MK-801
+ MK-801
- AβOs
+ AβOs
20 μm
B
p < 0.0001
% CCR positive neurons
30
20
10
0
AβOs - + - +
MK-801 - - + +
C
AβO time course -MK801
AβO time course +MK801
pCaMKII
total CaMKII
unt
15'
30'
1h
2h
D
p < 0.05
p< 0.0001
-MK-801
+MK-801
pCaMKII relative to total protein
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0:00
0:15
0:30
0:45
1:00
1:15
1:30
1:45
2:00
2:15
time (hours)

Figure 2: The NMDAR inhibitor, MK-801, blocks AβO-induced neuronal CCR and early activation of CaMKII. (A) Primary cortical neurons were treated overnight with AβOs with or without 10 μM MK-801. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that re-enter the cell cycle. (B) Quantification of the immunofluorescence results. Results have $p < 0.0001$ by one-way ANOVA. (C) Primary cortical neurons were treated for the indicated times with AβOs, with or without 10 μM MK-801, after which phospho-activated and total CaMKII levels were monitored by western blotting. (D) Quantification of phosphorylated CaMKII relative to total CaMKII at each time point. Note that MK-801 prevented the transient rise in phosphoactivation of CaMKII. Results are significant by two-way ANOVA ($p < 0.004$), and the 15-minute time point has $p < 0.0075$ by Bonferroni post hoc analysis.

3. Knockdown of NR1 blocks CCR

To provide independent, non-pharmacological evidence for the role of NMDAR in AβO-stimulated neuronal CCR, we used antisense shRNA to knock down (KD) expression of the constitutive NMDAR subunit, NR1. Primary neuron cultures were transduced with lentivirus containing an empty vector, or an shRNA vector to NR1, 96 hours prior to AβO addition. Following a 16-18 hour exposure to AβOs, the cells were processed for triple immunofluorescence with antibodies to MAP2, NeuN and cyclin D1. As shown in Fig. 3, we found that reducing the neuronal level of NR1 to 30% of normal blocked the ability of AβOs to cause CCR. These results validate the pharmacological evidence based on the use of MK-801 (Fig. 2) that NMDAR is an essential element of the molecular machinery that enables AβOs to drive normally post-mitotic neurons back into the cell cycle as a prelude to neuron death in AD.

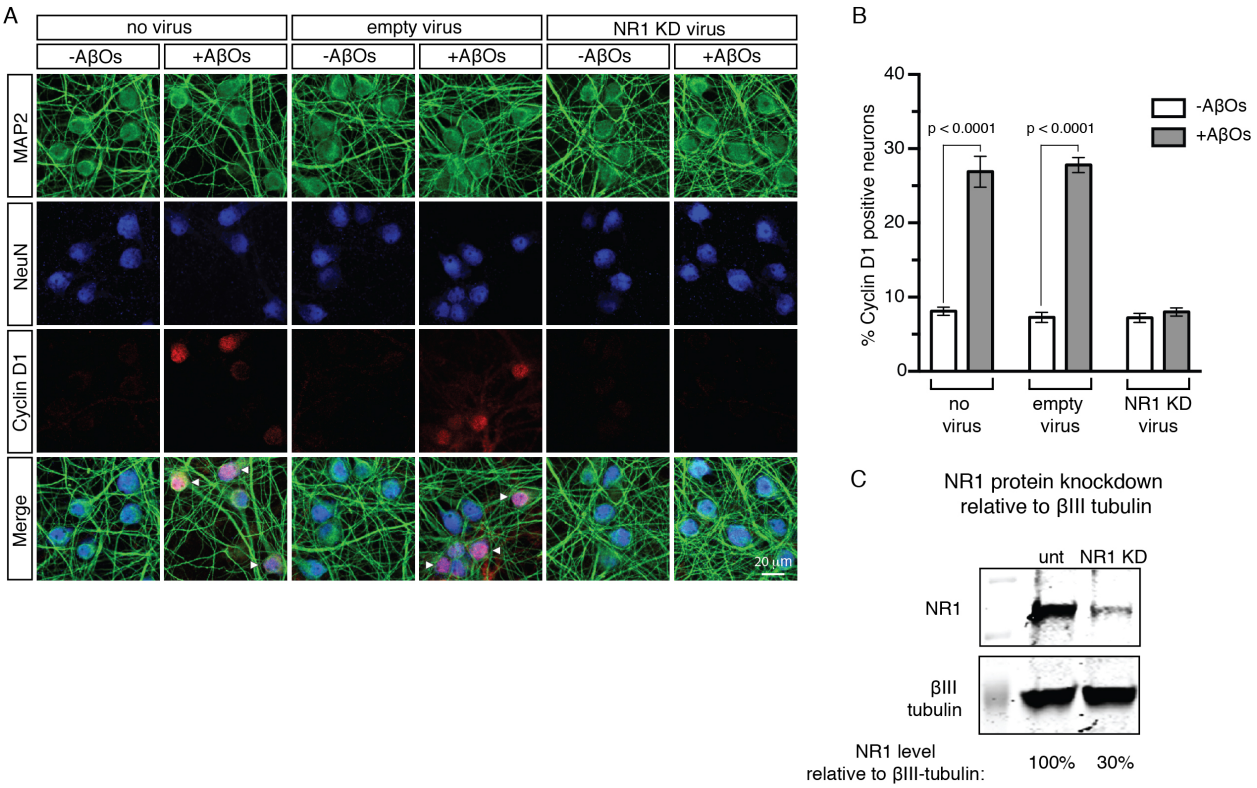


Figure 3: Knockdown of the NR1 subunit of NMDAR prevents AβO-induced neuronal CCR. (A) Primary cortical neurons were transduced for 96 hours prior to AβO addition with lentivirus expressing shRNA to NR1, or as a control, with lentivirus comprising an empty expression vector. After 16-18 hours of AβO exposure, the cells were stained by triple immunofluorescence for NeuN and MAP2 to mark neurons, and for cyclin D1 to assess CCR. (B) Quantification of the immunofluorescence results. Results are significant by one-way ANOVA with $p < 0.0001$. C) Quantitative western blot showing a 30% knockdown of NR1.

4. Inhibition of AMPA receptors by CNQX does not block CCR

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) plays an important role in the regulation of ion flux through NMDAR by allowing for depolarization of neurons in response to glutamate. This depolarization causes the displacement the magnesium ion from NMDAR and allows calcium influx through the channel pore. Given this role for AMPAR in the regulation of NMDAR calcium influx, we next tested whether AMPAR activation played a role in CCR induction. To test this possibility, primary neuron cultures were treated with 10 μM CNQX, a pharmacological inhibitor of the AMPAR, beginning 30 minutes before AβOs were added. After 16-18 hours of AβO treatment, neurons were fixed and stained for NeuN, MAP2 and cyclin D1 to enable quantitation of neuronal CCR. As shown in Fig. 4, treatment of neurons with AβOs in the presence and absence of CNQX led to a statistically significant increase in CCR. We therefore conclude that AMPAR does not play a role in the induction of CCR, and that rather in seems AβO-mediated calcium influx through NMDAR is AMPAR independent.

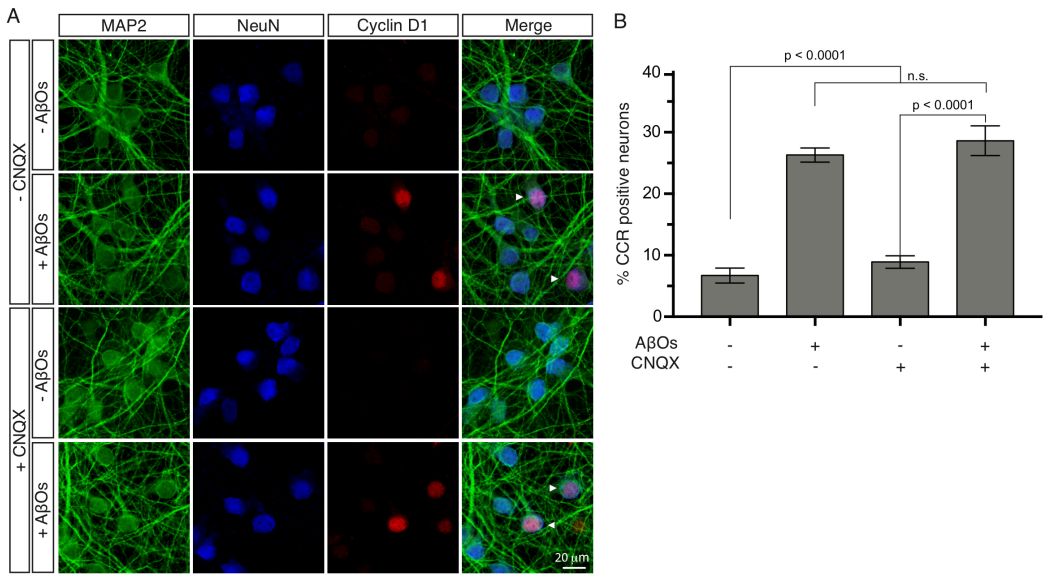
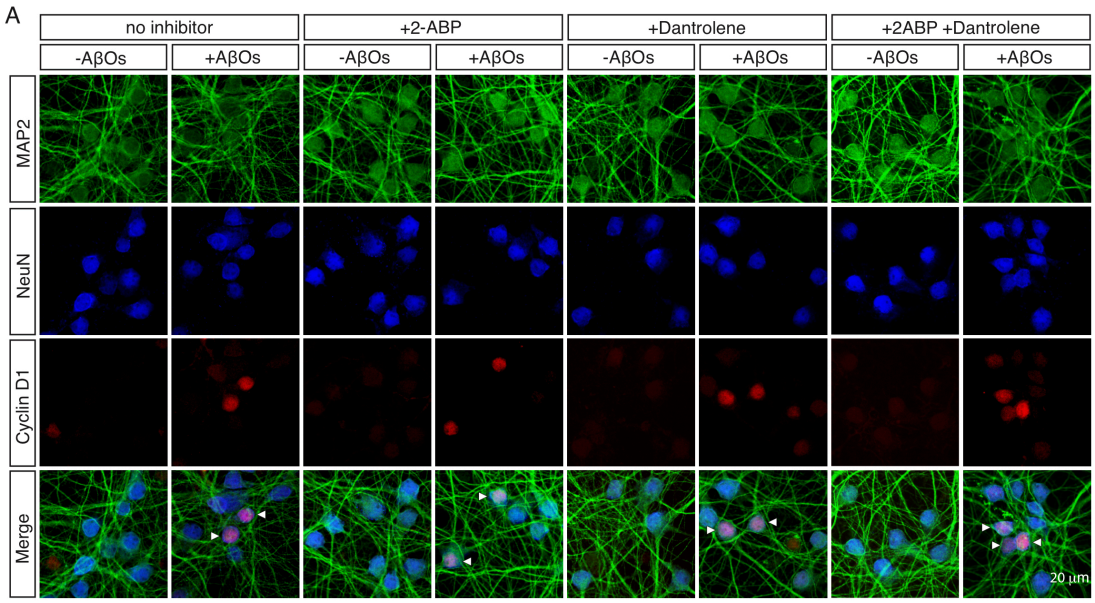


Figure 4: The selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor inhibitor, CNQX, does not inhibit CCR. (A) Primary cortical neurons were treated overnight with AβOs with or without 10 μM CNQX. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that re-enter the cell cycle. (B) Quantification of the immunofluorescence results. Both untreated neurons minus AβOs versus plus AβOs, and CNQX treated neurons minus AβOs versus plus AβOs, show significant increases in CCR ($p < 0.0001$ by one-way ANOVA) , indicating that AMPAR inhibition does not inhibit CCR from occurring.

5. The endoplasmic reticulum inhibitors to IP_3 and ryanodine receptors do not block CCR.

Experiments thus far have demonstrated a role for AβO-mediated calcium influx through NMDAR as being involved in neuronal CCR. However, in addition to calcium at the synapse playing a role in AD etiology, it has also been shown that calcium from endoplasmic reticulum is involved in calcium dysregulation in AD (Workgroup1, 2017). Additionally, initial calcium influx from NMDAR can cause calcium-induced calcium release, which could also contribute to the calcium dependence of CCR (Goussakov, Miller and Stutzmann, 2010). To test whether ER calcium is involved in the induction of CCR, we treated primary neuron cultures with either 50 μM 2-aminoethoxydiphenyl borate (2-APB) to block IP3 receptors and TRP channels, 10 μM dantrolene to block ryanodine receptors, or both inhibitors, 30 minutes prior to AβO treatment. After 16-18 hours of AβO treatment, neurons were fixed and stained for NeuN, MAP2 and cyclin D1 to enable quantitation of neuronal CCR. Regardless of the inhibitor treatment used, AβO treatment caused a statistically significant amount of CCR as marked by cyclin D1 positive neurons. This result shows that calcium from intracellular ER IP3 and ryanodine receptors are not involved in the induction of CCR. We therefore conclude that calcium from ER sources is not involved in the induction of CCR.


A
no inhibitor
+2-ABP
+Dantrolene
+2ABP +Dantrolene
-AβOs
+AβOs
MAP2
NeuN
Cyclin D1
Merge
20 µm

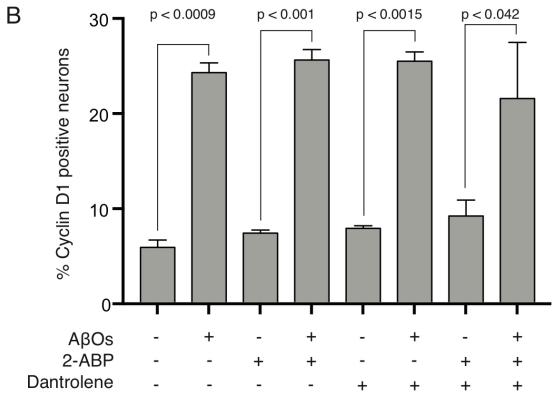

B
p < 0.0009
p < 0.001
p < 0.0015
p < 0.042
% Cyclin D1 positive neurons
30
20
10
0
AβOs - + - + - + - +
2-ABP - - + + - - + +
Dantrolene - - - - + + + +

Figure 5: The endoplasmic reticulum inhibitors to IP_3 and ryanodine receptors do not block activation of CCR. (A) Primary cortical neurons were treated overnight with AβOs with or without either 50 μM 2-aminoethoxydiphenyl borate (2-APB) to block IP_3 receptors, 20 μM dantrolene to block ryanodine receptors, or both inhibitors. After 18 hours of exposure to AβOs, neurons were stained for MAP2, NeuN, and Cyclin D1 to mark neurons that re-enter the cell cycle. (B) Quantification of the immunofluorescence results. In all drug combinations, there is a significant amount of CCR in samples treated with AβOs, showing that inhibition of ER calcium receptors does not inhibit CCR from occurring. Indicated p values were calculated by one-way ANOVA using the Bonferroni post-hoc test.

6. Memantine blocks CCR in cultured neurons

Memantine is an FDA-approved drug for AD treatment, and works by preventing excess calcium influx through NMDAR while still allowing normal calcium-mediated synaptic transmission through the receptor. To gain further insight into the mechanism by which excess calcium induces CCR, we tested whether specifically blocking excess, but not normal calcium entry through NMDAR could block CCR. Similar to experiments with MK-801, we treated primary neurons with AβOs for 16-18 hours with or without a 30 minute pre-treatment with memantine, followed by triple immunofluorescence labeling with antibodies to NeuN, MAP2 and cyclin D1. As shown in Fig. 4, memantine, like MK-801 (Fig. 2), prevented AβOs from inducing neuronal CCR. Although memantine is typically used to treat late stage AD and is not considered to be a disease-modifying drug, these results with cultured neurons indicate that memantine can interfere with the neuronal death pathway that is initiated by AβOs and is linked to CCR.

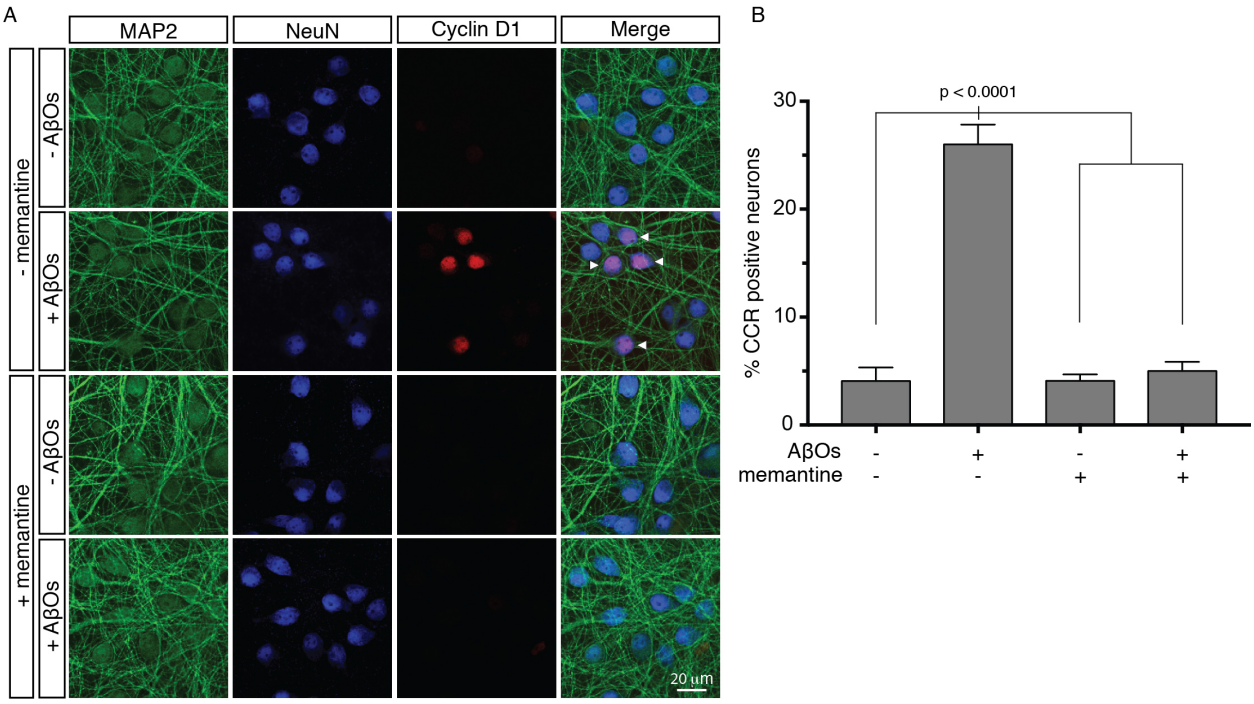


Figure 6: Memantine blocks AβO-induced neuronal CCR. (A) Primary cortical neurons were treated with AβOs with or without pre-treatment with 10 μM memantine. Cells were then stained with antibodies to MAP2, NeuN, and cyclin D1 to identify CCR neurons. (B) Quantification of the immunofluorescence results. Results are significant by one-way ANOVA with $p < 0.0001$.

7. Treatment of AD model mice with memantine prevents CCR

The effects of memantine on cultured neurons raised the prospect that the drug can modify disease progression *in vivo*. To test that possibility, wild type (WT) and Tg2576 AD model mice (Hsiao et al., 1996), which overexpress human APP with the Swedish mutation (K670M/N671L), were provided *ad libitum* access to memantine-containing water from the time they were weaned (3 weeks) until 2 months of age, when abundant neuronal CCR is normally evident in the Tg2576 strain (Norambuena et al., 2017). Following the 5 weeks of memantine treatment, the animals were euthanized and brain sections were stained with antibodies to NeuN, cyclin D1 and the neuron-specific protein, βIII-tubulin. As shown in Fig. 5, WT mice with or without memantine treatment had a basal level of 1.6% neuronal CCR along cortical regions. as determined by cyclin D1 immunoreactivity. In contrast, the basal level of CCR in similar cortical regions of Tg2576 mice was 8.8%, which was reduced to WT levels by memantine. These results show that treating Tg2576 mice with memantine before symptom onset acts prophylactically to prevent CCR.

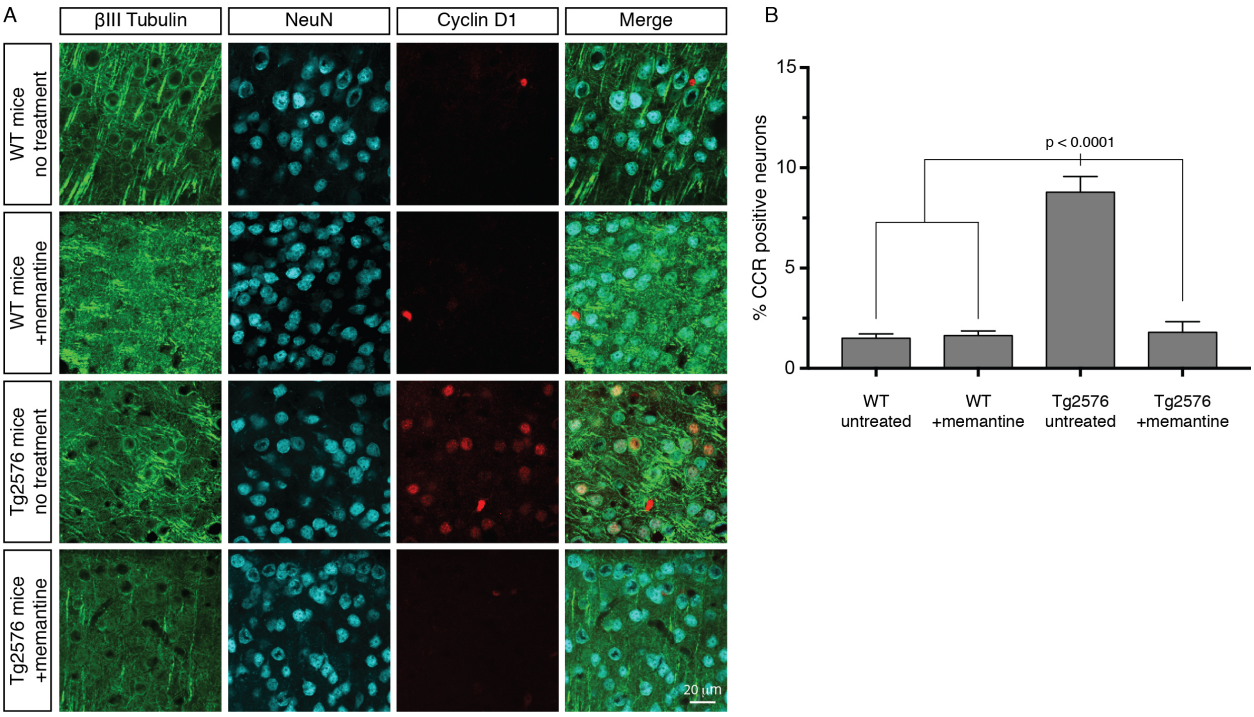


Figure 7: Treatment of Tg2576 AD model mice with memantine prevents CCR *in vivo.* (A) Tg2576 and wild type mice were provided *ad libitum* access to drinking water with or without memantine from the time of weaning at 3 weeks until 2 months of age. Brain sections from each condition were stained by triple immunofluorescence for the neuron-specific protein, βIII-tubulin, cyclin D1, and NeuN to identify CCR positive neurons. (B) Quantification of the immunofluorescence results.

8. *Model*

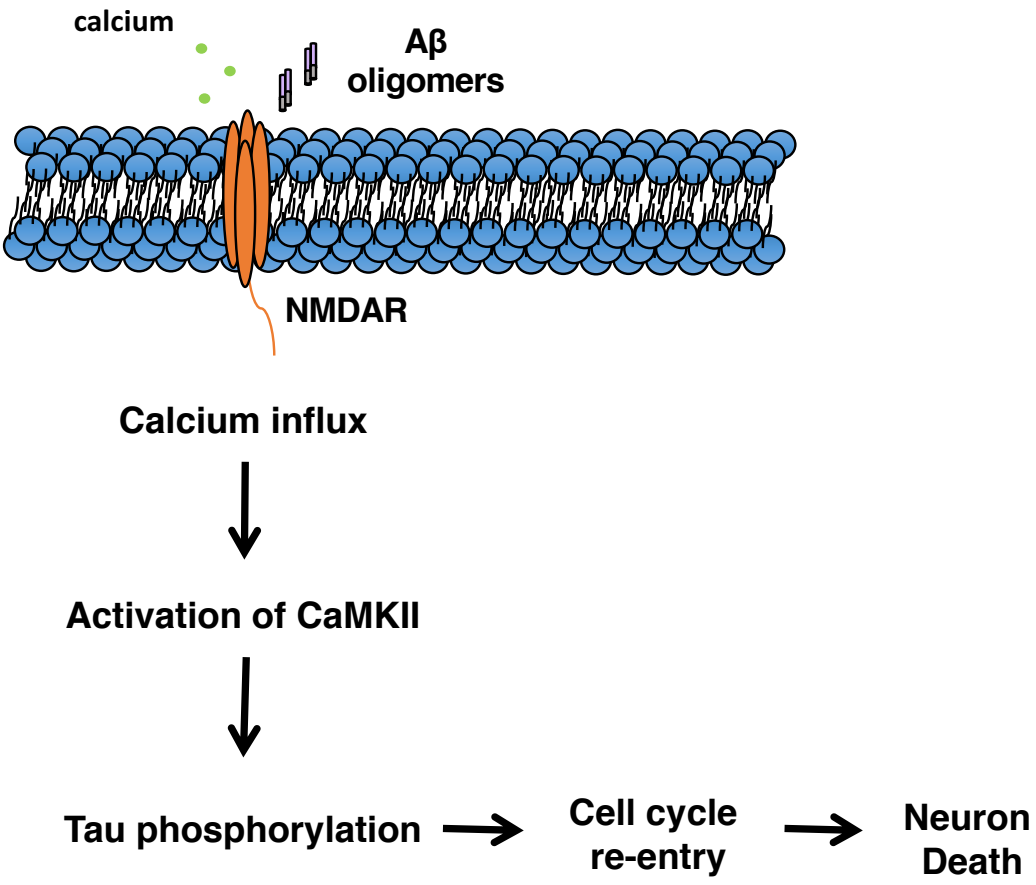


Figure 8: AβO-induced neuronal CCR is dependent on calcium disruption via NMDAR. Both CCR and calcium dysregulation contribute significantly to the pathogenesis of AD. Here, we describe a pathway in which AβO-mediated calcium entry induces neurons to re-enter the cell cycle, a pathway ultimately leading to the massive cell death seen in AD. Thus, AβO induced CCR is a calcium dependent process, specifically via NMDAR.

Chapter 3

Discussion and Future Directions

Discussion:

The work described in this thesis explores the role of excitotoxicity at the synapse and calcium disruption through NMDAR as a primary trigger for the onset of CCR, a read out of eventual neuron dysfunction and death in AD. We show for the first time that neuronal CCR is calcium dependent, and that NMDAR stimulation by AβOs is necessary for CCR. This AβO-mediated NMDAR stimulation also allows for the activation of CaMKII, a kinase known both for mediating excitotoxic effects of AβOs (Ghosh and Giese, 2015), as well as for phosphorylating tau at S416, a process necessary for CCR (Seward et al., 2013). Clinically, we show that treating the AD mouse model Tg2576 prophylactically with memantine, an FDA approved drug for treating AD that restricts calcium influx through the NMDAR channel pore, we are able to prevent CCR onset *in vivo*. Our work here demonstrates connections among NMDAR, excitotoxicity, and CCR, in that preventing excess calcium prevents CCR. But how are these pathways connected? The implications of these results and potential future directions of this research will be discussed in this section.

Excitotoxicity and the NMDA receptor have been implicated in numerous aspects of AD pathology. AβOs are the dominant species causing the excess calcium influx that contributes to excitotoxicity in AD, particularly through the NMDA receptor (De Felice et al., 2007; Zempel et al., 2010). In AD neurons, calcium dysregulation leads to myriad dysfunctions in the cell, eventually leading to their death. For example, NMDAR calcium influx at the synapse leads to increased production of reactive oxygen species causing oxidative stress, disruptions in LTD/LTP and synaptic function, which all contribute to

active signaling processes that ultimately lead to cellular death signaling. In addition to calcium dysregulation at the synapse, intracellular calcium stores can become disregulated and contribute to overall neuronal dysfunction in AD. Part of the role of intracellular calcium stores is to balance influx from the extracellular space, acting as a buffer to calcium levels in the neuron do not become too high or persist for too long. Thus, another calcium-mediated dysfunction in AD neurons is loss of control of this homeostasis. Although we have determined that calcium specifically from the activation of IP3 and ryanodine receptors on the ER does not contribute to CCR induction (Figure 5), we can not rule out that dysregulation of these calcium stores initiated by an NMDAR calcium influx is involved in CCR induction.

The fact that early AβO-mediated calcium influx via NMDAR is necessary to initiate CCR, before symptoms of necrotic cell death occur, is indicative of calcium playing a more active signaling role in this neuronal death paradigm. Supporting this is that neurons that have more recently entered the cell cycle look morphologically in tact, and do not show morphologic signs of a necrotic cell death. It is well documented, then, that CCR eventually leads to cell death, and that this is likely brought on by the activation of death signaling pathways (Herrup and Y. Yang, 2007a).

Even though this work focuses on the role of NMDAR-mediated calcium influx in causing CCR, it is worth exploring further which signaling pathways this calcium activates to initiate CCR. For instance, calcium released from intracellular sources oftens is involved in signaling for gene transcription at the nucleus, and is specifically implicated in gene transcription necessary to initiate cell cycle entry in regularly dividing cell types. For example, it was found in Swiss 3T3 cells that were serum starved and then

given back serum to induce CCR, that chelation of intracellular calcium inhibited both NF-κB activation and the transcription of cyclin D1, thereby inhibiting cell cycle induction (Sée et al., 2004). There could be a similar pathway controlling CCR in neurons, but that this CCR is initiating the death of these neurons rather than cell division. Even if intracellular calcium from the ER is not involved directly in mediating CCR, as is indicated by Figure 5, calcium signaling, especially from intracellular sources, has been shown to increase the amount of Aβ a neuron produces (Buxbaum et al., 1994; Querfurth et al., 1997). This would obviously contribute further to neuron dysfunction, CCR, and cell death, without being a link between calcium and CCR directly.

Are there other ways that calcium is connected to CCR? Calcium is one of the most versatile second messengers in many different cell types (Berridge, Lipp and Bootman, 2000), especially in the neuron, where calcium is vital for controlling multiple physiological processes. In fact, in cells that are normally dividing, calcium is known to be involved at multiple stages of the cell cycle. Two points of interest are that calcium by way of calmodulin is necessary to allow for a cell to come out of quiescence or G0, as well as at the transition from G1 to S phase (Chafouleas et al., 1984; Santella, 1998; Sée et al., 2004; Machaca, 2010). These transitions are especially important in terms of neuronal CCR, because it has been demonstrated that neurons undergoing CCR proceed through G1 to S phase and partially complete S phase, but never reach the cell division stage. Additionally, pharmacologic inhibition of CaMKII in mammalian cells blocks cells from going through the G1 to S transition (G. Rasmussen and C. Rasmussen, 1995; Tombes et al., 1995), and overexpression of calmodulin leads to increased proliferation (C D Rasmussen, 1987), showing a role for CaMKII in cell cycle related signaling

downstream of calcium influx. It could be that this already established pathway has been repurposed in the neuron as a response to stress or toxicity. Since calcium is such a fundamental signaling unit in the neuron, controlling multiple physiological processes for the cell, it makes sense that it would also be involved in toxic signaling paradigms as well.

Another potential link connecting these paradigms is tau. The role of tau at the synapse is being studied more in recent years, and tau has been implicated in numerous somatodendritic functions. The first connection between excitotoxic calcium influx and tau comes from the study of TKO mice. It is well established that reducing levels of endogenous tau in AD model mice and in cell culture is able to ameliorate many Aβ-induced deficits. Of particular interest to the results presented in this thesis is that TKO mice are also more resistant to excitotoxic seizures induced by kainate injection (Roberson et al., 2007). In a follow up to this study, it was also shown that reducing tau prevented synaptic and neuronal network impairment, as well as cognitive impairments in multiple human APP (hAPP) mouse lines (Roberson et al., 2011). Additionally, it has been shown that the NMDA receptor can be influenced by tau upstream of calcium influx, by recruiting Fyn to the synapse to phosphorylate the NMDA receptor, facilitating excess calcium influx and excitotoxicity by AβOs (Ittner et al., 2010). These studies support a role for tau at the synapse in both mediating excitotoxic calcium through NMDAR, as well as in mediating the effects of this calcium in the loss of synaptic integrity and subsequent neurodegeneration seen in AD. It is possible that this synaptic tau and calcium influx via NMDAR work together in the induction of CCR as well.

Work from our lab supports this theory further: we know from Seward, et al. that in TKO neurons, CCR does not occur, demonstrating that CCR is tau dependent. The work from Seward, et al. takes this tau dependence one step further, showing that the three kinases, CaMKII, PKA, and Fyn, need to be activated to initiate CCR, and that these kinases need to phosphorylate tau at three distinct epitopes (Ser416, Ser409, and Tyr18, respectively) for CCR to occur. The work in this thesis points to calcium influx via NMDAR being necessary for both CaMKII activation and subsequent CCR. Thus, although not unequivocally demonstrated, it is likely that NMDAR mediated activation of CaMKII is also responsible for phosphorylating tau at Ser416. These findings suggest one potential pathway connecting NMDAR, tau, and CCR, whereby tau is present at synapses and phosphorylated by CaMKII at Ser416 upon NMDAR calcium influx, which along with numerous other events, subsequently initiate CCR. Furthermore, low levels of tau have been shown to be localized to the somatodendritic compartment during regular synaptic transmission in neurons (Frandemiche et al., 2014), so it makes sense that tau could be at the synapse, presumably where these events occur. However, this is not the only possible explanation for these findings. Another interesting potential connection among CCR, NMDAR and calcium is the kinase Fyn. As mentioned previously, one somatodendritic function of tau is its recruitment of Fyn to NMDAR, where it modulates calcium coming through the channel. It's possible that Fyn phosphorylates tau while the two are bound together being recruited to NMDAR, and that something about this pathway is connecting them all to initiating CCR. What exactly about tau mislocalization, its binding to Fyn, or recruitment to NMDAR has to do with initiating CCR remains elusive, but is worth exploring further to better understand this pathway.

Rather than neurons exiting the cell cycle into G0 and staying post-mitotic from this point forward, it seems that keeping neurons post-mitotic is an active process, regulated by many cell cycle repressing proteins (Herrup and Y. Yang, 2007a). One of the key proteins responsible for holding neuronal cell cycle in check is Cdk5. This is demonstrated *in vivo* in mice knocked out for Cdk5, which show signs of neuronal CCR in areas of the brain that usually only contain post-mitotic neurons (Cicero and Herrup, 2005). It has also been shown in primary neurons that Cdk5 in the nucleus holds neurons in cell cycle arrest, and that translocation of this protein out of the nucleus results in neuronal CCR and cell death (Zhang et al., 2008). Under normal physiological conditions, the activation of Cdk5 is regulated by the activator protein p35 in neurons. Once Cdk5 is bound to p35, it can function to regulate various neuronal survival processes, such as those mediating neurite outgrowth and suppression of CCR. Despite the role of Cdk5 in neuron survival, in can also signal for cell death in various disease paradigms, including Alzheimer's disease and excitotoxicity. The switch of Cdk5 from physiological to pathogenic signaling appears to be regulated by the cleavage of the p35 activator by calpain, a calcium-dependent protease, to a p25 molecule that constitutively activates Cdk5. This chronically active form Cdk5, in turn, causes numerous dysfunctions in the neuron, including the mislocalization of Cdk5 to the cytoplasm, tau phosphorylation, and the induction of neurodegeneration and neuron death (Patrick et al., 1999; Z. H. Cheung and Ip, 2004). The above pathway is especially relevant to the data presented in this thesis, as a possible signaling pathway that connects AβO-mediated calcium influx through NMDAR with downstream activation of CCR, and importantly, neuron death. For example, AβO-mediated calcium influx via NMDAR could induce the

activation of calpain, which cleaves p35 into p25, and causes the dysregulation of Cdk5, inhibiting its role in keeping the neuron post-mitotic, inducing CCR and neuron death.

An important stride made toward combating the excitotoxicity contributing to synapse loss and cell death in AD patients is the drug, memantine. Memantine is an FDA approved drug used clinically to treat moderate to severe AD. It works by blocking excess calcium influx through the NMDA receptor channel pore (Lipton, 2006). Initial research with this drug in mice showed that myriad symptoms of AD are alleviated with treatment, including loss of LTP/LTD and learning and memory deficits (Minkeviciene, 2004) as well as synapse loss and abnormal phosphorylation of tau in AD (L. Li et al., 2004). Memantine is also protective against more canonic excitotoxic insults beyond AD—research in rats shows that when memantine is given either 1 hour prior to ischemic shock or immediately after the insult, damage to the CA1 region of the hippocampus is reduced (Seif el Nasr et al., 1990). Our results show that in addition to the ability of memantine to protect against synapse loss and behavioral deficits in mice, it can also work prophylactically to prevent CCR and subsequent cell damage if treated before symptom onset. Here, we take Tg2576 AD model mice, which overexpresses APP with the Swedish mutation causing an increase in soluble Aβ species, and treat them with memantine from the time of weaning until the onset of CCR. What we see is that memantine treatment prevents the onset of CCR in the mouse brain; in fact, memantine treated Tg2576 mice have no more CCR than WT controls. One of the biggest problems plaguing current AD treatments is that by the time drugs like memantine are given to patients, the disease has significantly progressed, and much of the damage that has occurred is irreversible. Our data provide evidence that at least one drug currently on the

market for the treatment of AD, memantine, might be an effective preventative treatments, in addition to its role in temporarily managing symptoms once they present. In order for this to be a viable option, however, we need faster and better diagnostic tools to be able to treat patients before permanent neuron damage occurs.

Research in this dissertation provides extensive evidence of Aβ-mediated calcium influx, which has long been implicated in mediating excitotoxic insults and synaptic deficits, also plays a role in initiating neuronal CCR. However, there are some potential conflicts and problems with these findings and other literature worth discussing. The first potential conflict comes from studies of CaMKII. There are some studies that show that there can be mislocalization of CaMKII from the synapse to the cytosol in AD affected neurons (Ly and W. Song, 2011; Reese et al., 2011). This is problematic in terms of the research presented in this dissertation, because we find that CaMKII activation is tied to calcium influx through NMDAR, which presumably puts CaMKII within the synaptic compartment. Furthermore, some researchers find a decreased level of activated CaMKII upon post-mortem analysis of AD brain (Amada et al., 2005), whereas we find that CaMKII activity is necessary for CCR pathology. However, I believe these discrepancies can be resolved by the timing of each of these events. For instance, in the results presented in this thesis, CaMKII activity occurs within 15 minutes of initial AβO exposure, whereas in both of the studies mentioned above, both subcellular redistribution and decreased activity are seen in patients with various stages of AD. It could be that in a healthy neuron, AβO-mediated calcium influx is one of the first pathogenic events in the disease, and that the chronic effects of this calcium influx over time induces the changes in CaMKII activity seen in AD patients. In fact, in the study by Reese, et al., (2011) they

see that increased mislocalization of CaMKII correlates with disease progression. This supports the idea that initially, calcium influx causes increased activity of CaMKII at the synapse, and over the course of disease progression its localization to the synapse and/or activated state is lost.

Another potential conflict at the synapse comes from studying examining the effect Aβ has on glutamatergic synaptic transmission and decreased synaptic plasticity. This study found that upon treatment of primary neuron cultures with AβOs, NMDA receptors are endocytosed within an hour of AβO treatment (Snyder et al., 2005). This endocytosis explains the deficiencies in synaptic transmission shown to accompany neuronal AβO treatment. Here, again, I think is a discrepancy in the timing of events. For example, AβO-induced calcium influx has been shown to occur within milliseconds of AβO exposure (De Felice et al., 2007; Alberdi et al., 2010; Zempel et al., 2010), with activation of CaMKII occurring before NMDAR endocytosis as well. Furthermore, I believe that this NMDAR endocytosis upon AβO exposure could be acting as a protective mechanism against AβO-induced excitotoxicity. It is possible that disruption of cellular mechanisms that regulate the levels of calcium in the cytoplasm, and sequester this calcium into ER and mitochondrial stores, induce NMDA receptors to be endocytosed to prevent more calcium from coming into the cell upon aberrant AβO-induced calcium influx.

A common rebuttal to CCR as a readout of neuronal dysfunction in Alzheimer's disease is that CCR is just a consequence of chronic stress in the neuron, rather than CCR representing a pathway that is activated to induce neuronal death. There are many

arguments against this line of thinking: that CCR occurs in multiple paradigms of cell death, including trophic withdrawal and excitotoxic shock, and that inducing CCR by inhibiting cell cycle repression by either Rb inhibition or knocking out Cdk5 induces death in neurons (Feddersen et al., 1992; E. Y. Lee et al., 1992; Cicero and Herrup, 2005). Even in the case that CCR is just a consequence of neurodegeneration in AD, it could still serve as a powerful diagnostic tool due to its early occurrence in disease progression.

Much of the research into neuronal CCR indicates both that keeping a cell in its post-mitotic state is an active process, and that dysregulation of this cell cycle inhibition eventually leads to neuron dysfunction and death. While there is much support for the induction of CCR during neuronal stress (mediated by AβOs or other insults), and that neurons that re-enter the cell cycle eventually succumb to death, the exact mechanism(s) connecting CCR to cell death once initiated remain elusive. Confounding the association between CCR and neuron death further is that multiple types of cell death are implicated in AD. Does CCR in the neuron only facilitate one particular death pathway, or is it more broadly applicable to neuronal catastrophe? Furthermore, is all neuron death in AD CCR dependent? And if not, what is the difference between CCR mediated and CCR independent cell death? More research is required into what connects CCR to neuron death before our understanding of ectopic neuronal cell cycle re-entry is complete.

Excitotoxicity mediated by AβOs in AD is widely accepted as playing a pivotal role in the etiology in AD: excess calcium floods the neuron, leading to the disruption of many signaling pathways, overloading synapses, ultimately leading to synapse loss and

stress on neurons early in the disease. These early symptoms contribute to the cell death later on in the disease, leading to the memory loss and cognitive decline characteristic of AD. We also know that neuronal CCR, one of the earliest symptoms of neuronal dysfunction in AD, over time, also leads to neuron death. The data presented here represents some of the first evidence to show that synaptic dysfunction and CCR are potentially connected, and that preventing AβO-mediated excitotoxicity and its downstream signaling also prevents neurons re-entering the cell cycle. Additionally, the *in vivo* memantine data shows that much of the damage that occurs in AD might be preventable by memantine, but that this prevention is may dependent on being able to diagnose AD before permanent damage occurs. A lot of what is known about the progression of AD focuses on single ailments and how they occur as a consequence of AβOs or toxic tau species accumulating in the brain. By focusing on connections between individual ailments, such as how calcium influences signaling allowing for CCR, we gain a better understanding of the disease as a whole, and move toward possible cures for AD.

Future Directions

The results presented in this thesis can be expanded upon in multiple ways to further elucidate the connections among excitotoxicity, CCR, and neuron death in AD. The first point of interest in expanding these results is in narrowing the specificity of Aβ-induced calcium influx. Though a role for NMDAR calcium influx has been well demonstrated in this thesis, it could be that a specific subset of NMDAR is responsible for initiating CCR. As mentioned in the Introduction, some research indicates that signaling from NR2B is pathogenic, resulting in synapse loss and contributing to neuronal dysfunction, while signaling from NR2A is neuroprotective. It would be interesting to see if blocking specifically NR2A or NR2B will also inhibit CCR, with the prediction being that only NR2B would block CCR onset. This experiment would be performed similar to the CCR assays performed in the results section. To block signaling from the receptors, viral shRNA vectors could be used to block NR2A and NR2B. There are pharmacological inhibitors for these receptors as well, though they are not as specific for their respective receptor subtypes as memantine and MK-801 are to NMDAR.

Another important detail that could be expanded upon in future research is the time frame in which calcium is important. Research presented in this thesis indicates that the first 15 minutes of Aβ exposure are necessary to active CaMKII, which is necessary to cause CCR. Is this the only time in which calcium is necessary? To test this, primary neuron cultures could be treated with Aβ for the first 15 minutes on their own, and after this time point, BAPTA-AM could be added to the media to chelate intracellular calcium. After 16-18 hours, cells could be stained for CCR markers. This would tell us if calcium

within 15 minutes is the only time calcium is involved in initiating CCR, or if calcium is also necessary is further downstream signaling. This experiment could also corroborate the results of the Snyder, et. al. 2005 study that shows in cultured neurons, NMDAR is endocytosed within an hour of Aβ treatment, which suggest that Aβ-mediated calcium influx through NMDAR could only be occurring initially in the CCR process.

Research in this thesis shows that NMDAR is necessary for initiating CCR, but could NMDAR also be sufficient for initiating this pathway? *In vivo* studies of excitotoxicity injury by kainic acid in mice suggest that activation of NMDAR on its own can induce CCR, but it would be interesting to test this hypothesis in primary neurons. In preliminary research not presented in this dissertation, I have found that treatment with NMDA continuously in the media kills neurons within an hour to two hours. Though these neurons do show signs of CCR in this short time frame, it is inconclusive whether this was an activation of a death pathway or aberrant gene expression occurring in severely damaged neurons. A better way to do this experiment would be to give neurons a 30 second excitotoxic shock with 50 μM NMDA such that they survive the insult, and then do a time course looking for CCR at time points ranging from 2 hours to 16-18 hours similar to when Aβ-mediated CCR occurs. This experiment would be useful in elucidating the role of NMDAR in CCR further, and whether NMDAR stimulation on its own is enough to induce CCR.

Another area of research worth pursuing in subsequent studies of CCR is the involvement of tau in this process. Previous research in the lab has shown that tau is necessary for CCR to occur. And while a lot of the subsequent research on this pathway

in the lab has provided insight into the mechanisms induced by Aβ that are involved in CCR induction, we do not know as much about what tau is doing in these networks to contribute to CCR.

Two points of interest with tau in the research presented in this dissertation is when tau is being phosphorylated by kinases known to be involved in CCR, and where tau is in the cell when this phosphorylation occurs. Research presented in this thesis shows that Aβ-mediated calcium influx occurs at 15 minutes, leading to CaMKII activation, and we know that CaMKII needs to phosphorylate tau at Ser416 to induce CCR. However, we do not know when this phosphorylation event on tau occurs, only that it is present when signs of CCR are present in the neuron. Knowing when this phosphorylation event on tau occurs could give further insight into the mechanism of this tau activation. This is true not only for CaMKII phosphorylation, but for PKA and Fyn phosphorylation as well, as we know those kinases play a similar obligatory role in CCR. To do this experiment, a time course similar to that shown for CaMKII activation in the results section could be done, where primary neurons are collected at various time points and run on a Western blot to check for when different phospho-epitopes are occurring.

Along this same line of thinking, it would be of great interest to know where tau was located once it has been phosphorylated, to narrow down potential interacting partners or signaling pathways tau is involved in that lead to CCR. For example, knowing that pS416 tau is present at the synapse could narrow down potential binding partners, or seeing tau at the nucleus could signify that it is involved in the transcription of cell cycle proteins. There are multiple ways of performing this experiment. One way would be to

purify synaptosomes or nucleosomes from the neuron, and look for changes in where tau is present after Aβ treatment. This experiment could also be done using microscopy, in either fixed cells at various time points, or by tagging tau with a fluorescent protein and following its movement over time with live cell imaging.

This experiment could also be done in the Tg2576 AD mouse line, collecting brains at various time points, starting with at least 2 months of age when CCR first occurs in these mice. With this experiment, the timing of tau phosphorylation would likely not be available, however, subcellular localization of pS416 tau could be determined in affected neurons. Furthermore, you could look for tau phosphorylation in various brain regions over time, to get an idea of how CCR spreads through these regions. This would provide more cohesive theory of how CCR spreads throughout the brain of an AD mouse model. Additionally, it would be interesting in the memantine treated mice to see whether tau phosphorylation at S416 is inhibited, to provide additional evidence that this phospho-tau site is mediated by NMDAR calcium influx.

Despite the extensive research done on neuronal CCR, there are still few studies that show a casual relationship between CCR and cell death. Many studies rely on correlative associations, such as neurons in areas with high rates of CCR also show cell death or a loss of neurons over time, or that CCR occurs in well established death paradigms, such as excitotoxic insult or growth factor deprivation. An experiment that could be done to show more strongly that CCR causes cell death would be to show that neurons that stain positive for CCR markers also show markers of cell death. As neurons *in vivo* can show signs of CCR long before neuronal death, the best way to do this experiment would be in cultured neurons, since the timing between CCR and neuron

death is more manageable. Furthermore, it would be important to use a permanent marker of cell cycle re-entry, such as BrdU incorporation, rather than a transient CCR marker such as cyclin D1. This way, if neuron death is occurring long enough after expression of CCR proteins, CCR and cell death could still be detected in the same cell. As there are multiple potential pathways by which neurons die in AD, using a non-specific cell death marker such as propidium iodine staining would be beneficial as well, to ensure dying neurons can be detected. It could also be interesting to use markers of specific types of neuron death, such as caspase staining, to narrow down the potential ways in which CCR acts to induce neuron death.

The last proposed experiment comes from the experiment showing that treatment of Tg2576 mice with memantine prevents CCR *in vivo*. This result indicates that taking a drug such as memantine before neuron damage occurs can be neuroprotective, preventing the onset of CCR and other behavioral deficits in mouse models. It seems as though it is worth testing the possibility that memantine can act prophylactically in humans as well as in mice. The major problem plaguing AD treatment currently is that by the time drugs such as memantine are given to patients, massive neuronal damage and death may have already occurred in the brain. Even with the modern advances in diagnosing this disease, it is hard to be able to diagnose someone with AD before they present with the memory deficits characteristic of the disease. However, there are two populations of individuals that are more prone to developing AD, and it could be worth running a clinical trial in these population subsets. The first population are those with familial mutations in APP and PS1 that are 100% penetrant for the development of Alzheimer's, though these cases make up a very small percentage of all AD cases. The second population of individuals

are those who are carriers of the APOE-4 allele. This population presents a slightly better cohort for testing the preventative effects of memantine for two reasons. One, not everyone who is APOE-4 positive will end up having the disorder, so negative controls are built into the study. Secondly, these cases represent a sporadic form of the disorder that plagues the majority of patients, so if there are differences between familial and sporadic onset, this population would account for these differences. Admittedly, this is not an experiment that could be done in our current lab setting. However, as a clinical trial I believe this idea has a lot of merit.

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