

**Impairment of Fast Axonal Transport
in Alzheimer's Disease**

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THESIS

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LIST OF ABBREVIATIONS

5xFAD	Transgenic mouse with five FAD-linked mutations
AAA	ATPase Associated with diverse cellular Activities
Ach	Acetylcholine
AchE	Acetylcholinesterase
AchEI	Acetylcholinesterase Inhibitor
AD	Alzheimer's disease
ADAM	A Disintegrin And Metalloproteinase
ADRDA	Alzheimer's Disease and Related Disorders Association
AICD	APP Intracellular Domain
ALS	Amyotrophic Lateral Sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
AMP-PNP	Adenosine 5'-(β,γ -imido)triphosphate
APH	Anterior Pharynx Defective
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
ATP	Adenosine triphosphate
A β	Amyloid beta
BACE	β -site APP Cleaving Enzyme
CamKIIa	Calcium/Calmodulin dependent kinase IIa
CDK5	Cyclin-Dependent Kinase 5
cDyn	Cytoplasmic Dynein
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CK2	Casein Kinase 2
CNS	Central Nervous System
CSF	Cerebro-spinal Fluid
DHC	Dynein Heavy Chain
DIC	Dynein Intermediate Chain
DIV	Days in Vitro
DMAT	2-Dimethylamino-4,5,6,7-Tetrabromo-1H-benzimidazole
DS	Down Syndrome
FAD	Familial Alzheimer's Disease
FAST	Functional Assessment Staging Test

LIST OF ABBREVIATIONS (continued)

FAT	Fast Axonal Transport
FDA	Food and Drug Administration
FTDP-17	Frontotemporal Dementia and Parkinsonism linked to chromosome 17
GSK-3b	Glycogen Synthase Kinase 3b
HD	Huntington's Disease
HEK cells	Human Embryonic Kidney cells
HSP	Hereditary Spastic Paraplegia
IP	Immunoprecipitation
JNK	c-Jun N-terminal Kinase
KHC	Kinesin Heavy Chain
KLC	Kinesin Light Chain
LC	Dynein Light Chain
LIC	Dynein Light Intermediate Chain
MBOs	Membrane Bounded Organelles
MCI	Mild Cognitive Impairment
MMSE	Mini-mental Status Examination
MRI	Magnetic Resonance Imaging
MT s	Microtubules
NFT	Neurofibrillary Tangle
NIA	National Institute of Aging
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMDA	N-methy-D-aspartate
PD	Parkinson's Disease
PEN	Presenilin Enhancer
PHF	Paired Helical Filament
PKA	Protein Kinase A
PKC	Protein Kinase C
PMCN	Primary Mouse Cortical Neurons
PP1	Protein Phosphatase 1
PS	Presenilin
PS1-ki	Presenilin 1 knock-in
SAD	Sporadic Alzheimer's Disease
SAPK	Stress Activated Protein Kinase

LIST OF ABBREVIATIONS (continued)

SCa	Slow Component-a
SCb	Slow Component-b
SLO	Streptolysin O
SN	Supernatant
SPM	Spermine
SYP	Synaptophysin
TBCA	Tetrabromocinnamic Acid
WT	Wild Type

SUMMARY

Alzheimer's disease (AD) is the most common adult-onset neurodegenerative disease, and it affects over 5 million Americans. AD is also the most common cause of dementia, the loss of cognitive functions, among the elderly. Pathological characteristics of AD include the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles, and significant loss of cholinergic neurons in hippocampus and basal forebrain. The cause of AD is largely unknown except for the genetically inherited familial form of AD which mutations in proteins such as Presenilin 1 (PS1), PS2, and amyloid precursor protein (APP) have been identified. Like other neurodegenerative diseases, only neurons are affected in AD although pathological proteins are ubiquitously expressed in many different types of tissues. Why neurons degenerate while other cell types remain normal is not fully elucidated.

Neurons are different from other cell types in that they have highly polarized structure with an axon which can be several thousand times the length of the cell body. There is little to no protein synthesis in the axon which means all the materials required for proper functioning of axon and its terminals must be transported from the site of their synthesis, the cell body in anterograde transport machinery. Also, signaling molecules and materials to be degraded are transported retrogradely in the axon. Cytoskeletal and soluble proteins are transported in the slow component of axonal transport, and fast axonal transport (FAT) transports membrane bounded organelles (MBOs). MBOs are transported in the anterograde direction by kinesins and in the

SUMMARY (continued)

retrograde direction by dyneins. Neurons' high dependence on axonal transport for their functions and survival makes them more vulnerable to any insult that disrupts transport than other types of cells.

Recently, the role of impaired FAT has been implicated in the pathogenesis of a number of neurodegenerative diseases, including AD. Moreover, a dominant negative mutation of anterograde motor Kif5A is linked to Hereditary Spastic Paraplegia (HSP) Type 10, a progressive neurodegenerative disease that affects lower motor neurons (Reid et al, 2006). However, mutations in motor proteins themselves are rare, and no such mutation is linked to AD. An alternative explanation is that pathological proteins affect FAT's regulatory processes. FAT is regulated by phosphorylation of motor proteins. Aberrant kinase and phosphatase activities can lead to untimely release of cargoes or abnormal transport velocities which will result in dying back neurodegeneration.

In AD, hyperphosphorylation of tau protein provides a clear evidence for dysregulation of phosphotransferase activities. One of the kinases shown to have altered activity in AD is CK2, a pleiotropic Serine/Threonine kinase (Iimoto et al., 1990). Perfusion of isolated squid axoplasm with active CK2 inhibits both directions of FAT (Morfini et al., 2001). However molecular mechanism of this inhibition and its role in AD has not been previously studied. In this thesis, a detailed analysis of molecular events caused by elevated CK2 activity leading to impairment of

SUMMARY (continued)

FAT and their implications in the pathogenesis of AD are described. First, FAT velocity experiments with perfusion of the oligomeric A β 42 (oA β 42) which is the major component of amyloid plaques, a pathological hallmark of AD, revealed oA β 42 directly activates CK2 and impairs both directions of FAT. Second, molecular mechanisms of FAT disruption by CK2 were studied using biochemical approach. Microtubule (MT) binding assays with primary mouse cortical neurons with CK2 activator revealed kinesin's binding to MT is reduced upon activation of mouse endogenous CK2. A novel mechanism of alteration in dynein-based retrograde transport by CK2 activation was also identified. Iodixanol vesicle flotation assays failed to detect changes in motor protein binding to MBOs upon CK2 activation, but further study is needed to determine subpopulation specific effect of CK2.

Studies using primary cortical neurons of a mouse model of AD (PS1-ki M146V) demonstrated that both kinesin and dynein binding to MT are reduced in this transgenic mouse line while binding to the MBOs were not affected. Previous studies suggest kinesin light chains are more phosphorylated at CK2 consensus site although alteration in CK2 activity was not detected in permeabilized cortical neuron culture from this animal in current work. Very aggressive mouse model of AD (5xFAD) exhibited brain region specific activation of CK2. These results lead us to present a novel mechanism of the pathogenesis of AD which increased production of A β 42 leads to altered activation of CK2 which impairs FAT causing neurons in specific brain region degenerate in dying back fashion.

SUMMARY (continued)

To further understand late-onset nature of AD, additional studies revealed that expression levels of kinesin are developmentally regulated. With less kinesin available at later age, neurons are especially vulnerable to dysregulation of regulatory processes of FAT such as abnormal activation of CK2.

CHAPTER I

1. INTRODUCTION AND OVERALL HYPOTHESIS

Adult-onset neurodegenerative disease Alzheimer's disease (AD) is characterized by presence of extracellular plaques mainly composed of amyloid beta ($A\beta$) peptides, intracellular neurofibrillary tangles (NFT) with its main component being abnormally phosphorylated microtubule binding protein tau, and neuronal atrophy. Etiology of AD is not clearly elucidated except for few percent of genetically inherited cases of early-onset familial-type AD (FAD) where mutations are found in proteins such as presenilin 1 and 2 (PS1 and PS2), and amyloid precursor protein (APP). Although these mutations are present since birth, clinical symptoms do not appear until adulthood. These proteins are ubiquitously expressed in all cell types (Kovacs et al., 1996; Uhlen et al., 2005), but cell damages in AD are restricted to certain cell types. Neurons in the hippocampus and basal forebrains are targeted by the disease, while other neuronal populations such as neurons in the cerebellum are relatively spared. Because of their unique morphology, neurons are highly dependent on the transport system between cell body and axon terminals. Impairment of axonal transport can result in dying back neuropathy. Fast axonal transport (FAT) which transports membrane bound organelles (MBOs) is tightly regulated by activities of phosphotransferases. Activities of kinases such as GSK3 β and CK2 are shown to be altered in brain samples of AD patients and in brains of transgenic mice carrying FAD-linked mutations. Phosphorylation of motor protein kinesin light chain (KLC) by GSK3 β negatively affects anterograde FAT, however effects of CK2 activation on FAT are not fully understood.

I propose that FAT is impaired in AD in part due to abnormal activation of protein kinase CK2. Experiments in this dissertation will show that oligomeric A β and FAD-linked PS1 mutations cause alterations in CK2 activities. CK2 impairs FAT by reducing motor protein binding to both its tracks, microtubules (MTs), and to its cargoes, MBOs. Further, dysregulation of time and region specific activities of CK2 combined with unique expression patterns of motor proteins lead to cell type specific vulnerability in AD.

2. Specific Aims

Aim 1. To evaluate effects of oligomeric A β 42 on fast axonal transport.

Amyloid beta (A β) peptide is a product of proteolytic cleavage of APP. Two main species of A β are A β 40 and A β 42 which are 40 and 42 amino acids in length, respectively. A β 42 is more amyloidogenic compared to A β 40, and is the main component of the amyloid plaques. It is believed that A β 42 exist in at least three different conformations; monomeric, soluble oligomeric, and fibrillar. The oligomeric A β 42 (oA β 42) is considered as an intermediate conformation in fibrillogenic process (Parbhu et al, 2002). Historically fibrillar A β 42 has been viewed as neurotoxic, but mounting evidence now suggests that oA β 42 is the neurotoxic conformation of A β 42 (Dahlgren et al, 2002). The molecular mechanism on how oA β 42 exerts its effect is still not known. *I propose that oA β disrupts FAT, a critical process for neuronal function, via activation of protein kinase CK2.* The experiments in this aim examine the effects of oA β 42 and altered protein kinase activities on FAT using isolated squid axoplasm.

Aim 2. To investigate molecular mechanisms of CK2 activation on FAT impairment.

Phosphorylation of kinesin heavy chain by certain kinases may result in inhibition of kinesin binding to microtubules, whereas phosphorylation of kinesin light chain may result in release of MBOs from kinesin. As a result, phosphorylation of either component of kinesin may decrease the number of selective MBO transported in anterograde axonal transport.

Phosphorylation of dynein might similarly affect retrograde transport. These phosphorylations may happen as normal functions of these kinases in the cell or as pathological conditions.

Several kinases have been implicated in phosphorylation of kinesin and/or cDyn. Proper functioning of the axonal transport system requires all of those kinases involved to function accordingly. It has been shown GSK3 β selectively phosphorylates KLCs and cause MBOs to dissociate from kinesin (Morfini et al., 2002b). How CK2 decreases kinesin- and cDyn-based FAT has not been investigated in a mammalian system. *I propose that CK2 phosphorylates motor proteins and impairs FAT by reducing their binding both to MT and to MBOs.* The experiments in this aim would address questions of whether phosphorylation of kinesin and cDyn by CK2 will reduce their binding to MT and to MBOs.

Aim 3. To examine impairment of FAT in animal model of AD.

Dysregulation of kinases has been widely reported in patients and animal models of AD. For example, mutations in cytosolic loop of PS1 increase interactions between PS1 and GSK-3 β , and activity of GSK-3 β is elevated in non-neuronal cells transfected with mutant PS1 (Takashima et al, 1998; Pigino et al, 2000). These increases in GSK3 β activity were correlated with reductions in synaptic vesicles in axons and axon terminals of mutant PS1-ki mice (Pigino et al, 2003). *In vivo* nerve ligation study also suggests that FAT is impaired in sciatic nerve of

FAD linked PS1 mutant mice (Lazarov et al, 2007). However, molecular events underlining the impairment of FAT in these animal models have not been studied extensively. Several animal models of AD have genetic mutations that lead to production of higher level of A β 42, which may favor the formation of more oA β 42. *Here I propose that kinesin- and cDyn-based vesicle motility is impaired in neurons of mutant human PS1-ki mice due to over activation of CK2 in a region specific manner.* Combination of region specific dysregulation of CK2 and developmentally regulated expression of motor proteins contribute to the unique cell type specific pathology of AD. The experiments in this aim examine whether the impairments of FAT in FAD linked PS1-ki mice are mediated by increased activities of CK2. In addition, experiments examine region and time specific expression of kinesin heavy chain isoforms.

CHAPTER II

LITERATURE REVIEW

1. Alzheimer's Disease

1.1. History of Alzheimer's disease

Alzheimer's disease (AD) was first described by Dr. Alois Alzheimer, a German psychiatrist and neuropathologist, on November 4, 1906 at the conference of South-West German psychiatrists. He gave a presentation about his patient, 51-year-old Auguste D, the wife of an office clerk, who had been institutionalized with a list of symptoms such as reduced comprehension and memory, disorientation, and paranoia. Clinical notes Alzheimer made of Auguste during her first few day of institutionalization were recently discovered and translated into English by Drs. Maurer, Volk, and Gerbaldo (Maurer et al., 1997). The file contains a conversation between Alzheimer and Auguste, and it shows the severity of Auguste's memory impairment. For example, (*italics denote Auguste's answer*) "What is this? I show her a pencil. A *pen*. A purse and key, diary, cigar are identified correctly. At lunch she eats cauliflower and pork. Asked what she is eating she answers *spinach*. When she was chewing meat and asked what she was doing, she answered *potatoes* and then *horseradish*. When objects are shown to her, she does not remember after a short time which objects have been shown." (Maurer et al., 1997) Auguste's condition deteriorated quickly, and she died on April 8, 1906 due to sepsis caused by infected bedsores. Upon post-mortem examination of Auguste's brain, Alzheimer described "neurofibrils which can be distinguished by their unique thickness and capacity for impregnation" and "the deposition of a special substance in the cortex" (Stelzmann et al., 1995).

These later became known as neurofibrillary tangles and amyloid plaques, respectively, two pathological hallmarks of AD. Alzheimer later published these findings in 1907 in an article titled “Über eine eigenartige Erkrankung der Hirnrinde – On an unusual illness of the cerebral cortex.” The name “Alzheimer’s disease” was first used by Alzheimer’s colleague Emil Kraepelin, a German psychiatrist, in 1910 in the 8th edition of *Handbook of Psychiatry*.

1.2. Patient demographics

According to the Alzheimer Association, in 2010, an estimated 5.4 million Americans have AD, and 5.2 million of them are age 65 and older (www.alz.org/facts, Alzheimer Association, 2012). Although about 65% of elderly patients are female, this is due to the fact that females live longer than males. When this is taken into consideration, females are no more likely to develop AD than males at any given age. The educational (Plassman et al., 2007) and racial background of an individual (Dilworth-Anderson et al., 2008) have an effect on the prevalence of AD. However, it is not clear if this is due to a direct causal relationship or confounding factors such as discrepancies in accessibility to health care and a higher prevalence of other conditions (i.e. high blood pressure and diabetes) that can increase the risk of developing AD. With the older population growing, it is predicted that the number of AD patients will increase to 13.2 million in the United States by 2050 (Hebert et al., 2003).

1.3. Clinical symptoms and diagnosis of Alzheimer’s disease

The symptoms of AD can be categorized into cognitive and non-cognitive symptoms. Cognitive symptoms include impairments in memory, language, and decision making. Disorientation with time and place is also common. For example, a patient may misplace a phone

in the refrigerator and have no memory of it even after he or she finds it. Likewise, a patient may get confused and get lost in a very familiar place. Examples of non-cognitive symptoms are agitation, aggression, depression, apathy, delusion, and hallucination. Among these, depression and apathy are two of the most common non-cognitive symptoms (Lyketsos et al., 2011).

The diagnostic criteria of AD were initially established by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA, a former body of Alzheimer's Association) in 1984 (McKhann et al., 1984). In 2011 the National Institute on Aging (NIA) and the Alzheimer's Association published revised diagnostic criteria to accommodate discoveries and advances made in the past 27 years (Jack et al., 2011). In the revised criteria, AD is divided into three stages of the disease: 1) preclinical AD, 2) mild cognitive impairment (MCI) due to AD, and 3) dementia due to AD. These revisions acknowledge that AD-related pathological changes occur well before the symptoms appear and that the disease progresses in a continuum scale. Also, the revisions recommend establishing tests for biomarkers such as blood and cerebral spinal fluid (CSF) tests to identify changes in the level of disease related proteins as well as neuroimaging like magnetic resonance imaging (MRI) to detect brain atrophy. Some tests for biomarkers are showing promising results in predicting progression from MCI to AD (Palmqvist et al., 2012). However, because the biomarker tests lack widely accepted standards for determining the presence of the disease (McKhann et al., 2011), they mainly serve a research purpose and not a significant component of clinical diagnosis at this time. Currently, diagnosis of AD is made from interviews with the patient and people around him or her, the patient's medical and family history, and batteries of cognitive and functional tests. These tests include the clock drawing test (Sunderland et al., 1989) the mini-mental status exam (MMSE)(Folstein et al., 1975), and

functional assessment staging test (FAST) (Reisberg, 1988), as well as any tests necessary to eliminate other diseases that can possibly cause cognitive impairment. A patient with “MCI due to AD” progresses to “dementia due to AD” as cognitive and behavioral symptoms become severe enough to interfere with the patient’s ability to function in daily life.

According to the new diagnostic criteria, a patient does not have to be clinically diagnosed with dementia to receive a pathological diagnosis of AD in the post-mortem brain autopsy. Pathological diagnosis is made using “ABC staging protocol.” “A” stands for distribution of amyloid plaques, the “B” parameter ranks a brain according to the neuropathological staging method for neurofibrillary tangle established by Braak, H and Braak E (Braak and Braak, 1991), and “C” is neuritic plaque assessment using the standards from the consortium to establish a registry for Alzheimer’s disease (CERAD) (Mirra et al., 1991). Each criterion is assigned to four stages from 0 to 3 depending on the extent of pathology. “0” is absence of the pathology and “3” is extensive presence of the pathology. Using these staging methods, a report will be produced as “Alzheimer Disease Neuropathologic Changes: A2, B1, C1” or “Alzheimer Disease Neuropathologic Changes: A3, B3, C3” (Hyman et al., 2012).

1.4. Brain pathologies in Alzheimer’s disease

A human cerebral cortex can be divided into the frontal, temporal, parietal, and occipital lobes, and each of them is associated with different functions. The occipital lobe, for example, is responsible for processing visual information, and the frontal lobe plays an important role in executive functions such as problem solving, attention, and working memory. The temporal lobe, especially the medial temporal lobe, contains the hippocampus and is critical for memory formation (Squire and Zola, 1996). In AD, the frontal and the temporal lobes are more severely

affected while the occipital lobe is relatively spared. In 1953, Mr. Henry Molaison (H.M.), 29-year-old motor worker, went under an operation to remove his medial temporal lobe bilaterally in an attempt to correct epileptic seizures he suffered from for many years. The surgery was successful in its primary objective to reduce seizure severity, and a battery of tests revealed that his intelligence, perception, abstract thinking, reasoning ability, and motivation were unchanged or slightly improved compared with pre-surgery assessment. However, his memory and ability to form new memories had been severely affected (Scoville and Milner, 1957). The research community gained an immense amount of understanding of the relationship between the medial temporal lobe and memory from cases like Mr. Molaison's and several other patients who underwent similar surgeries leading to comparable impairments in memory.

In AD, the most notable macroscopic brain pathology is brain atrophy. Upon post-mortem brain examination, the overall volume of AD brains is significantly reduced compared to non-AD brains, with the largest difference in temporal lobe (Mann, 1991). One of the major causes of this atrophy is the selective loss of cholinergic and glutamatergic neurons in the basal forebrain and entorhinal cortex, which are closely connected with the hippocampus (Davies and Maloney, 1976). The basal forebrain is the major source of cholinergic projections in the central nervous system (CNS), and has important roles in the sleep-wake cycle, attention, learning, and memory. The entorhinal cortex is the main relay station between the hippocampus and the neocortex, and plays a major role in memory and learning. In these neurons, synaptic impairment is the initial sign of dysfunction. There is a significant reduction in the number of synapses in biopsied frontal and temporal cortices from AD patients (Davies et al., 1987) and the levels of the synaptic marker protein, synaptophysin, in post-mortem brains with AD (Masliah et al., 2001) when compared with age-matched cognitively healthy brains. Widening of the sulci (the

grooves), narrowing of the gyri (the ridges), and enlargement of ventricles are seen concomitantly with brain atrophy (de la Monte, 1989; Forstl et al., 1995). The rate of brain atrophy is well correlated with disease progression (Fox and Freeborough, 1997).

In addition to brain atrophy, two pathological hallmarks of AD are the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). The amyloid plaques are insoluble fibrous aggregates of protein fragments mainly composed of A β peptides (Glenner and Wong, 1984). A β is a 39~43 amino acid peptide that results from proteolytic cleavage of Amyloid Precursor Protein (APP), an abundantly expressed integral transmembrane protein encoded by a gene on Chromosome 21 (Kang et al., 1987). Two major species of A β are A β 40 and A β 42, which are 40 or 42 amino acids long, respectively. Both species of A β are found in amyloid plaques, but due to its hydrophobic sequences, A β 42 is more amyloidogenic (Kim and Hecht, 2006). Genetic mutations that cause an inheritable form of AD have been shown to increase A β 42 to A β 40 ratio by either reducing production of A β 40 or increasing production of A β 42 (Bentahir et al., 2006). Other genetic mutations increase overall processing of APP to increase levels of A β .

While amyloid plaques are found in extracellular spaces, the NFTs on the other hand are found inside the neurons. NFTs are insoluble protein aggregates formed around a core of paired helical filaments (PHFs) made of abnormally phosphorylated tau, a microtubule (MTs) associated protein (Kosik et al., 1986). Tau is mainly expressed in neurons, and six isoforms due to alternative splicing are identified in the human central nervous system. Multiple isoforms of tau are present in NFTs, and they are all abnormally phosphorylated (Goedert et al., 1992). The presence and distribution of NFTs are better correlated with AD progression than amyloid plaques. The NFTs appear in the transentorhinal cortex of the temporal lobe initially and spread

to other parts of the brain as the disease progresses. The group of neurodegenerative diseases that are characterized by the presence of NFTs such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Spillantini et al., 1998), and dementia pugilistica (Roberts, 1988) are called “tauopathies”. AD is the best known and researched example of the tauopathies.

1.5. Treatment of Alzheimer’s disease

Despite extensive research, currently there is no cure for AD, and no treatment to reverse the neurodegeneration or stop progression of the disease. Acetylcholinesterase inhibitors (AChEI) and N-methyl-D-aspartate (NMDA) receptor antagonists are two types of drugs that are used to ameliorate the symptoms of memory impairments. Acetylcholine (ACh) is a neurotransmitter that is produced by cholinergic neurons and plays an important role in learning and memory in the CNS. ACh is synthesized in cholinergic neurons by choline acetyltransferase from choline and acetyl-Co-A, and rapidly degraded by acetylcholinesterase in the synaptic cleft. In AD brains, levels of ACh receptors are significantly reduced in the hippocampus and the nucleus basalis of Meynert in the basal forebrain (Shimohama et al., 1986). The administration of an ACh receptor antagonist in rats produced memory impairments similar to AD (Bymaster et al., 1993). During neurotransmission, ACh is released into the synaptic cleft from the presynaptic terminal, binds to the ACh receptors on the postsynaptic terminal membrane, and initiates signal transmission. ACh has a very short half-life, and it is quickly degraded by Acetylcholinesterase (AChE). AChEIs such as Donepezil, Rivastigmine, and Galantamine suppress AChE activity, thus increasing the amount of ACh available to bind to receptors (Rogers and Friedhoff, 1996) and improve neuronal signal transmission. The effectiveness of AChEIs on AD symptoms varies among individuals, but at best they only delay disease progression for up to 12 months.

In 2003, Food and Drug Administration (FDA) approved Memantine, a NMDA receptor antagonist, to manage moderate to severe AD symptoms. In addition to the Ach, glutamate is another important neurotransmitter in the CNS that plays a role in learning and memory (Mukherjee and Manahan-Vaughan, 2012). Glutamate binds to NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainate receptors as well as metabotropic receptors (mGluR) on the postsynaptic membrane to modulate synaptic plasticity. However, overstimulation of these receptors causes an excessive influx of calcium (Ca^{2+}) into the neurons, activating a number of enzymes and eventually leading to apoptosis by excitotoxicity. In AD, excitotoxicity seems to play a role in dysfunction of the glutamatergic neurons (Hynd et al., 2004). Memantine is a low affinity voltage-dependent non-competitive antagonist of NMDA receptors, and it prevents excitotoxic damage to the neurons by inhibiting prolonged influx of Ca^{2+} without altering the receptor's function (Molinuevo et al., 2005). Memantine has been shown to be effective in slowing the progression of cognitive deterioration (Hellweg et al., 2012), but it still does not stop progression or reverse the damage of AD.

1.6. Etiology of Alzheimer's disease

1.6.1. Familial Alzheimer's disease

The two types of AD are familial AD (FAD) and sporadic AD (SAD). FAD is caused by mutations in genes that encode for APP, presenilin 1 (PS1), or presenilin 2 (PS2), and is inherited in an autosomal dominant fashion. There are over 30 mutations on the APP gene that are linked to FAD. The gene that encodes for APP is located on chromosome 21, and the protein products vary in length from 695 to 770 amino acids due to alternative splicing. The cellular function of APP is not known although roles in synaptogenesis (Torroja et al., 1999),

neuronal migration (Herms et al., 2004), and regulation of cerebral blood flow (Koike et al., 2012) have been suggested. APP is subjected to many post-translational modifications such as glycosylation (Pahlsson et al., 1992), phosphorylation (Suzuki et al., 1992), and sulfation (Walter and Haass, 2000). The best known and characterized post-translational modification is proteolytic cleavage by α , β , and γ -secretases that produce different protein fragments. In non-amyloidogenic processing, APP is cleaved by α -secretase (A Disintegrin And Metalloproteinase (ADAM) 9, 10, and 17) to produce soluble APPs α and membrane-bound C83 fragments. The C83 fragment can be further cleaved by the γ -secretase complex to generate a soluble P3 fragment, leaving the APP intracellular domain (AICD) embedded in the membrane. The exact composition and actions of γ -secretase complex are not yet fully understood, but the γ -secretase complex involves PS1 or PS2, nicastrin, presenilin enhancer 2 (PEN2), and anterior pharynx defective (APH1) (Kaether et al., 2006). The amyloidogenic pathway involves both β and γ -secretases. B-secretase (β -site APP-cleaving enzyme 1; BACE1) cleaves APP first to release a soluble APPs β and C99 fragment. The γ -secretase complex then cleaves C99 to produce AICD and the A β peptide (LaFerla et al., 2007). FAD-linked mutations in APP are concentrated around the β and γ -secretase cleavage sites, and many of them have been shown to increase amounts of the more amyloidogenic A β 42 (Scheuner et al, 1996). Interestingly, Jonsson and colleagues recently reported that a mutation in APP at the amino acid 673 protects against AD and cognitive decline in the elderly (Jonsson et al., 2012). In the same study, this mutation also lead to an approximately 40% reduction in the production of A β peptide compared to WT APP in human embryonic kidney (HEK) cell lines.

PS1 and PS2 are highly homologous proteins encoded by PSEN1 on chromosome 14 and PSEN2 on chromosome 1, respectively. Over 130 mutations in PS1 and 15 mutations in PS2

have been linked to FAD (Cruts et al., 2012). The presenilins are multi-pass transmembrane proteins which function as the catalytic component of the γ -secretase complex that cleaves APP to generate A β peptides (Wolfe et al., 1999; Zhang et al., 2000). Soon after they are translated, presenilins undergo endoproteolytic cleavage that separates the protein into 27-28 kDa N-terminal and 16-17 kDa C-terminal fragments (Thinakaran et al., 1996). In addition to their role in amyloidogenesis, proteolytic activity of presenilins is also important in Notch signaling (De Strooper et al., 1999) as well as a wide range of other substrates (Haapasalo and Kovacs, 2011; Lleo, 2008; Wolfe, 2010). The Notch signaling pathway is critical for cell to cell communication and regulation of embryonic development, and mice deficient in PS1 are embryonically lethal with severe deformation of skeleton and CNS (Shen et al., 1997). The γ -secretase independent functions of presenilins such as regulating calcium homeostasis and autophagy have been suggested (Neely et al., 2011; Supnet and Bezprozvanny, 2011), but they remain controversial (Shilling et al., 2012; Zhang et al., 2012). Mutations in presenilins are randomly distributed, but clustered in transmembrane alpha helix regions (Dillen and Annaert, 2006). Similar to mutations in APP, FAD-linked mutations in the presenilins also increase production of A β 42 (Scheuner et al., 1996, Citron et al., 1997).

People with FAD-linked mutations develop AD before the age of 60, some as young as in their 30s. However, FAD is clinically and pathologically indistinguishable from SAD. FAD only accounts for less than 5% of total cases of AD, and the rest of the cases are sporadic forms.

1.6.2. Sporadic Alzheimer's disease

Sporadic AD is the most common form of the disease. Age of onset for SAD is 65 and older, and advancing age is the biggest risk factor for AD. Dietary lifestyle (Ho et al., 2004),

metal ions such as mercury (Hock et al., 1998) and aluminum (McLachlan et al., 1996), and herpes simplex virus type 1 (HSV1) (Wozniak et al., 2009) have all been implicated in the pathogenesis of AD, but no clear relationships have been established. A genetic factor, the apolipoprotein E allele, has been identified to increase the risk of developing AD.

Apolipoprotein E (ApoE)- ϵ 4 significantly increases the risk of late SAD (Corder et al., 1993).

The three isoforms of human ApoE are ApoE 2, 3, and 4, and ApoE3 is the most common isoform. While allele ϵ 4 increases the risk, allele ϵ 2 lowers the risk of AD. The primary source of ApoE in the central nervous system is astrocytes. Manelli et al. (1972) showed, using enzymatic activity of mitochondria as an output, that the presence of ApoE4 expressing astrocytes exacerbates the cellular toxicity of oligomeric A β 42 (Oa β 42) on neurons expressing mouse ApoE (Manelli et al., 2007). However, it is not clear in this study if ApoE4 produced and secreted by astrocytes is the direct cause of neurodegeneration, or if something else secreted by astrocytes that is unique to ApoE4 expressing cells leads to neurodegeneration.

1.6.3. Down syndrome and Alzheimer's disease

Down syndrome (DS) is caused by complete or partial trisomy of chromosome 21. Normally, an individual has two copies of chromosome 21, but people with DS have three copies of either full or part of this chromosome (Lejeune et al., 1959). People with DS suffer from mental retardation, hypotonia, and a high incidence of congenital heart defects. In addition, DS individuals develop AD by the time they reach 40 years of age, and show histological characteristics of AD at an early age (Wisniewski et al., 1985). One theory for the propensity of people with DS to develop AD is that the additional chromosome has another copy of APP. It has been reported that mere duplication of APP is sufficient to cause AD (Sleegers et al., 2006),

but the mechanisms of how overexpression of APP leads to dementia and neurodegeneration have not been elucidated.

1.7. Hypotheses for the pathogenesis of Alzheimer's disease

1.7.1. Cholinergic hypothesis

The cholinergic hypothesis is that AD manifests due to reduced synthesis and release of acetylcholine (ACh). This hypothesis was supported by a report showing selective loss of over 75% of acetylcholine producing neurons in basal forebrain of AD brains (Whitehouse et al., 1982). In AD brains, the level of choline acetyltransferase, ACh uptake by neurons, and release of ACh from post-mortem brain tissue have all been shown to be reduced compared to the normal brains (Perry et al., 1977; Rylett et al., 1983); Nilsson et al, 1986). A class of drugs that inhibits acetylcholinesterase was developed based on this hypothesis. They have been shown to ameliorate some symptoms of AD however they have not been successful in halting the disease nor reversing the clinical and pathological damage. This hypothesis has largely fallen out of favor and more recent hypotheses focus on the pathological hallmarks of AD: tau and amyloid plaques.

1.7.2. Tau hypothesis

The tau hypothesis states that NFT is the primary cause of clinical and pathological manifestations of AD. It is based on the fact that the extent and localization of NFT pathology are well correlated with AD symptoms (Braak and Braak, 1991). Tau binds to and stabilizes a major cytoskeletal protein in neurons MTs. Experiments suggested that abnormally phosphorylated tau has reduced ability to bind to MTs, and this would lead to destabilization and

degradation of neurons' cytoarchitecture (Biernat et al., 1993). It is not clear; however, if the phosphorylation promotes detachment of tau from MT, or phosphorylation of tau is caused by detachment of tau and formation of NFT (Mudher and Lovestone, 2002). Further, loss of microtubules does not appear to be an early or primary event in AD. Mutations in a gene encodes for tau have been linked to adult-onset dementia such as FTDP-17, but no mutations have been linked to AD.

1.7.3. Amyloid hypothesis

The original amyloid hypothesis states that amyloid plaques mainly composed of A β 42 lead to activation of microglia and astrocytes, initiating a cascade of inflammatory responses in the CNS. As a result, neuronal ionic homeostasis becomes disturbed. This eventually leads to degeneration of a selective population of neurons, which manifests as dementia. This hypothesis was formulated based on observations that people with DS, who have triplication of APP which A β peptides are derived from, develop AD in their 40s (Wisniewski et al, 1985). Additionally, AD brains exhibit numerous amyloid plaques. However, the number and regional distribution of amyloid plaques in the brain does not correlate well with cognitive impairment in AD (Terry et al., 1991), and high amyloid burden is sometimes observed in the brains of cognitively healthy elderly subjects (Driscoll et al., 2006). Similarly, inflammation is variable in AD and efforts to treat AD by anti-inflammatories have not been every effective. This led to a revision of the amyloid hypothesis that smaller, soluble A β aggregates may play a role in the early stages of AD pathogenesis. Intracellular oligomeric A β localizes at synaptic terminals (Kokubo et al., 2005), and is associated with synaptic (Ishibashi et al., 2006) and cognitive dysfunction (Cleary et al,

2005). The revised amyloid hypothesis still fails to provide molecular mechanisms to explain how A β leads to the selective neurodegeneration of neurons and AD.

1.7.4. Transport Hypothesis

Diagnosis of AD requires presence of both the amyloid and the tau pathologies, and neither pathology is sufficient to cause the disease by itself. This led to formulation of a hypothesis that these pathologies affect a cellular process dependent or independent of each other which eventually leads to selective degeneration of neurons. In AD, many proteins associated with the disease are expressed in cells other than neurons (Joachim et al., 1989; Lee et al., 1996), but neurons are selectively affected. This suggests that AD is caused by the disruption of a cellular process that is uniquely important to neurons. Neurons vary in size and the type of signals they carry depending on their location and function; however, neurons consistently have three major structural specializations. The cell body, or soma, contains the nucleus, and protein synthesis occurs almost exclusively here. Protruding from cell body are small-branched processes called dendrites that receive input and constitute the postsynaptic terminal of the synapse with a small amount of protein synthesis. The third specialization is a long tube like structure called the axon that effectively lacks protein synthesis. Unlike dendrites which can number in the hundreds per cell, each neuron has only one axon. In adult humans the axon of the sciatic nerve or a corticospinal neuron can extend for a meter or more. At the distal end of the axon are the presynaptic terminals, where neurotransmitters are released and outflow of information occurs. With its unique morphology and lack of protein synthesis machinery within the axon, the neuron heavily relies on a transport system to move materials such as neurotransmitters, growth factors, mitochondria, ion channels, and lipids anterogradely from the

cell body to presynaptic terminals and retrogradely from presynaptic terminal back to the cell body (Grafstein and Forman, 1980). Various studies have suggested that disruption of axonal transport is closely associated with AD (Lazarov et al., 2007; Morfini et al., 2002a; Pigino et al., 2003; Smith et al., 2007)

2. Axonal Transport

Neuron is a fundamental component of the vertebrate nervous system. Because of its unique morphology and because most protein synthesis occurs in the cell body (Campenot and Eng, 2000), large amounts of protein need to be transported between the cell body and the synaptic terminals as well as everywhere in between. Moreover, the axon has multiple domains which differ in structures and functions. For example, voltage gated sodium channel 1.6 is localized at nodes of Ranvier to facilitate timely signal conduction (Caldwell et al., 2000), and receptors for neurotransmitter are enriched in synaptic terminals. This spatial heterogeneity of the axon is maintained by a highly regulated transport system in which materials are continuously and selectively transported to the appropriate destinations in a timely manner. Axonal transport is a critical cellular process for neurons to survive and to function, playing a much more critical role in neurons than comparable transport processes in non-neuronal cells.

2.1. Types of Axonal Transport

2.1.1. Slow Axonal Transport

Axonal transport is divided into two types according to transport velocities; slow and fast. Slow axonal transport transports non-membrane bound proteins such as cytoskeletal proteins

(Griffin et al, 1978) and cytosolic proteins (Paggi and Petrucci, 1992) at a rate of 0.1-20mm/day. It is further divided into slow component-a (Sca) and slow component-b (SCb) according to its rate, 0.1-1mm/day and 0.2-8mm/day, respectively (Lasek et al., 1984). Sca is associated with cytoskeletal proteins such as microtubule and neurofilaments. SCb is associated with cytoplasmic protein such as Calcium/Calmodulin dependent kinase (CamKIIa) and synapsin (Scott et al., 2011). In its early days, study of axonal transport involved radio-labeling proteins in neurons and track the movement of “peaks” over time (Hoffman and Lasek, 1975). These peaks strongly suggest that proteins are actively transported rather than being diffused. It also revealed that different groups of cytosolic proteins move at different velocities. Unlike the FAT which moves more persistently with fewer stops, the motors in the slow axonal transport make frequent stops in between the short burst of rapid movement (Roy et al., 2007). This intermittent mode of transport results in reduced net velocity.

2.1.2. Fast Axonal Transport

FAT represents the transport of MBOs such as synaptic vesicles, mitochondria, and lysosomes at various rates ranging from 50 -400 mm/day. The anterograde FAT (from cell body to the cell periphery and synaptic terminals) are associated with transport of synaptic vesicles, mitochondria, and axolemmal components needed for maintenance and function of neurons (Brady, 1985; Vale et al., 1985). MBOs containing trophic factors, exogenous materials, and old membrane components are transported in retrograde direction from cell periphery and synaptic terminals to the cell body (Paschal et al., 1987; Sobreviela et al., 1996). The molecular mechanisms and regulation of FAT have been studied more extensively compared to that for the slow axonal transport which has been difficult due to technical issues (Roy et al., 2005). Imaging

techniques such as Video Enhanced Contrast Differential Interference Contrast and time-lapse microscopy (Allen RD 1985, Timmer et al, 2009) have been widely used for the study of FAT. Other methods of studying FAT include ligation of the sciatic nerve and evaluate the accumulation of axonally transported proteins (Lazarov et al., 2007), manganese enhanced MRI (Van der Linden et al., 2002), and other biochemical assays.

2.2. Motor proteins

In FAT, MBOs are transported at 200-400mm/day in anterograde (Brady et al., 1985) and at 100-250mm/day in retrograde direction (Susalka et al., 2000) along the microtubule (MT) tracks. The MTs which are long, hollow cylindrical polymers of alpha and beta tubulin subunits are the main cytoskeletal proteins that make up the axons. MTs have intrinsic polarity, and they are uniformly oriented in the axon. A faster growing plus end is pointed towards the terminal and a slow growing minus end is pointed towards the cell body (Heidemann et al., 1981). This polarity provides the basis for the directionality of motor proteins which transport cargo along MTs.

2.2.1. Kinesins

Anterograde transport from the cell body towards the terminal is carried out by kinesins which are encoded by Kif genes. There are 45 mammalian Kif genes, and 15 families based on the sequence homology (reviewed in Hirokawa et al, 2009). The most abundant kinesin motor is kinesin 1 or Kif5s, which was the first one to be discovered and shown to be enriched in the nervous system (Brady, 1985; Vale et al., 1985). Kinesin 1 exists as a tetrameric holoenzyme composed of homodimer of 110-134 Kda heavy chains (KHCs) and two 60-70 Kda light chains

(KLCs) (Bloom et al., 1988; DeBoer et al., 2008). There are three heavy chain genes (kinesin-1 A, B, and C) and two or more light chain genes which undergo alternative splicing (Cyr et al., 1991). Kinesin 1 heterotetramer has two globular heads, a stalk, and a fan-like end when observed by electron microscopy with a total length of 80nm (Hirokawa et al., 1989). KHCs contain microtubule binding and ATPase (motor) head domain, tail domain that interacts with light chains, and hinged stalk domain in between. Kinesin uses the energy from ATP hydrolysis to generate mechanical force to move towards the plus end of MTs. The light chains interact with heavy chains in amino terminus, and bind to vesicles/cargoes in carboxyl terminus (Hirokawa et al., 1989). There is a region of 15 heptad repeats in the amino terminus which are predicted to form an alpha helical coiled-coil structure (Cyr et al., 1991). This region is thought to be responsible for dimerization and interaction with the KHCs. Four imperfect tandem repeats of 42 amino acids in the carboxyl terminus of KLCs contain groups of hydrophobic and charges residues, and this is thought to mediate KLC binding to the MBOs (Cyr et al., 1991). Indeed, a monoclonal antibody against this region inhibited FAT by interfering kinesin binding to the membrane vesicles (Stenoien and Brady, 1997). In addition to the binding to the MBOs, highly heterogeneous carboxyl terminuses of KLCs are thought to be responsible for targeting kinesin 1 to specific cargos (Wozniak and Allan, 2006).

2.2.2. Dyneins

Neurons also depend on retrograde transport of MBOs containing degradation materials and signaling vesicles such as activated neurotrophin receptors from cell periphery and axon terminals back to cell body for their function and survival. The motor protein responsible for retrograde transport is a minus end-directed microtubule-based motor cytoplasmic dynein (cDyn)

(Schroer et al., 1989). It is a 1.2Mda multisubunit protein complex consisting of two heavy chains of 530Kda (DHCs), two 74Kda intermediate chains (DICs), four 53-59Kda light intermediate chains (LICs), and several light chains of ~8Kda (LCs) (Dick et al., 1996; Susalka and Pfister, 2000). Carboxyl terminus of the DHC contains six AAA (ATPase Associated with diverse cellular activities) modules, and four of them include nucleotide-binding sites (P-loops) (Takahashi et al., 2004b). The cDyn utilizes the energy from ATP hydrolysis in the P-loops to move on the MT tracks. DHC also contains MT binding domain in the carboxyl terminus between AAA4 and AAA5 (Koonce, 1997). The amino terminus, tail domain of DHC contains self-association, and DIC and LIC binding sites (Habura et al., 1999). The DICs binds to the cargo, and their alternative spliced isoforms are thought to be responsible for specificity of cargo targeting (Nurminsky et al., 1998; Susalka et al., 2000). CDyn also interacts with multiprotein complex, dynactin (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin consists of p150Glued, p135Glued, p62, dynamitin, actin-related protein 1 (Arp 1), actin, actin-capping protein α and β subunits, p27, and p24. Dynactin directly interact with cDyn through binding of p150Glued with DICs (Vaughan and Vallee, 1995). The p150Glued is the major subunit of dynactin and loss of function mutation of this protein in mice results in early lethality with abnormal accumulation of cytoskeletal and synaptic vesicle proteins at neuromuscular junctions (Lai et al, 2007). Dynactin has been proposed to increase the processivity of cDyn by enhancing MT-cDyn binding and to regulate ATPase activity of cDyn (King and Schroer, 2000; Kumar et al., 2000).

2.3. Fast axonal transport impairment and dying back neuropathy

The volume and length of axons relative to the cell body puts immense demand on proper functions of FAT for the health and maintenance of the neuron. When transport between cell

body and presynaptic terminal is compromised, proteins needed to maintain and promote neuronal survival do not get when and where they are needed. This may cause synaptic failure because molecules such as receptors for various neurotransmitters that are required to maintain synaptic connections with other cells do not get delivered to presynaptic specializations, and eventually neuronal cell death occurs as the endpoint of a dying back neuropathy (Morfini et al., 2009a).

The importance of the FAT and motor proteins has been highlighted recently in several studies of missense mutations in motor proteins and neurodegenerative diseases. Autosomal dominant mutations in kinesin1A (Kif5A) motor domain have been linked to both early- and late-onset hereditary spastic paraplegia which is characterized by progressive spasticity in the lower limbs (Reid et al, 2002, Blair et al, 2006). Also, mutations in dynactin subunits have been linked to distal spinal and bulbar muscular atrophy and Perry syndrome (Puls et al, 2005, Farrer et al, 2009). Despite the clear relationships between the FAT and etiology of some neurodegenerative diseases, occurrence of mutations in motor proteins is rare. An alternative explanation for impaired FAT in neurodegenerative diseases is dysfunction in the regulatory mechanisms of the FAT.

2.4. Regulation of fast axonal transport

FAT is tightly regulated through activities of phosphotransferases such as protein kinases and phosphatases. Conventional kinesin exist as phosphoprotein in vivo (Hollenbeck, 1993), and phosphorylation of kinesin correlates with organelle binding and neurite outgrowth (Lee and Hollenbeck, 1995) and transport of insulin containing β -granules (Donelan et al., 2002). Studies from our and other laboratories revealed that both heavy and light chains of kinesin contains

numerous consensus sites for phosphorylation by various kinases, and phosphorylation of some of the residues results in alterations of FAT in squid axoplasm. Phosphorylation of the heavy chains result in dissociation of kinesin from MTs, and phosphorylation of the light chain releases cargoes from the motor (Morfini et al., 2001). For example, c-Jun N-terminal kinase (JNK) phosphorylates KHC, and this reduces kinesin binding to the MTs (Morfini et al., 2006). Glycogen synthase kinase 3 β (GSK3 β) selectively phosphorylates KLCs and inhibits kinesin 1 based anterograde FAT by promoting the release of kinesin from MBOs (Morfini et al., 2002b). Moreover, some protein kinases and phosphatases can affect the kinesin binding to MBOs indirectly. For example, the inhibition of cyclin-dependent kinase 5 (CDK5), a neuronal specific protein kinase, results in inhibition of anterograde FAT in squid axoplasm (Morfini et al., 2004). However, CDK5 does not directly phosphorylate kinesin. Instead, inhibition of CDK5 activates protein phosphatase 1 (PP1) which in turn activates GSK3 β . The activities and localization of GSK3 β and CDK5 are shown to be regulated during neuron maturation (Hur and Zhou, 2010; Nikolic et al., 1996). This suggests precise control of kinase activities is crucial for the delivery of specific cargoes to specific axonal locations at precise timing.

Regulation of cDyn function is not well understood. Yet, studies revealed that nearly all polypeptide isoforms of cDyn are phosphorylated at serine residues *in vivo*, and phosphorylation of cDyn regulates its function (Dillman and Pfister, 1994; Whyte et al., 2008). It was also found that extent of DHC phosphorylation differs between inactive and active state (Dillman and Pfister, 1994). Although the molecular mechanism of the effect has not been fully elucidated, perfusion of PKC catalytic subunit in squid axoplasm increased cDyn mediated retrograde FAT (Morfini et al., 2007). Moreover, inhibition of serine/threonine protein phosphatases increased phosphorylation of DHCs and DICs which lead to reduced ATPase

activity of cDyn (Runnegar et al., 1999). These data strongly suggest that function of cDyn is tightly regulated by phosphotransferase activity.

3. Fast axonal transport and Alzheimer's disease

Altered kinase and phosphatase activities have been observed in a variety of neurodegenerative diseases such as Parkinson's disease (PD) (Smith et al., 2003), Huntington's disease (HD) (Khoshnan et al., 2004), and Amyotrophic Lateral Sclerosis (ALS) (Holasek et al., 2005). Proteins related to these diseases have also been shown to affect FAT. Micromolar level of mutant alpha-synuclein that causes some hereditary forms of PD reduces anterograde and increases retrograde FAT in squid axoplasm (data not published). Furthermore, perfusion of polyglutamine expansion disease protein at concentration >100 fold lower than molecular motors inhibits both directions of FAT significantly (Szebenyi et al., 2003). This suggests that disease related proteins impair normal FAT by affecting enzymatic pathway rather than the direct sequestration of motor proteins (Morfini et al., 2009b).

In AD, changes in GSK-3 β (Pigino et al., 2003) Yamaguchi et al, 1996), stress activated protein kinases (SAPK) such as P38 and JNKs (Hensley et al., 1999), PKA (S. H. Kim, Nairn, Cairns, & Lubec, 2001), PKC (Alkon et al., 2007), and CK2 (Blanquet, 2000; Perez et al., 2011; Pigino et al., 2009) activities have been reported. All of the kinases above have been shown to phosphorylate kinesin, and all but PKA alter the rate of FAT in one or both directions. Impairments of FAT in AD have also been reported in animal models of the disease. Hippocampal neuronal culture of mutant PS1 knock-in mice showed reduced immunoreactivity

of syntaxin which is anterogradely transported by kinesin, and synaptophysin, a synaptic vesicle marker, in axon (Pigino et al., 2003). Sciatic nerves of the mice harboring FAD linked PS1 mutation show impaired anterograde fast axonal transport of selected membrane proteins (Lazarov et al., 2007). Considering the complexity of FAT regulation involving multiple kinase and phosphatase pathways, it is plausible that dysregulation of phosphotransferase activity leads to dying back neurodegeneration due to impaired FAT.

CHAPTER III

MATERIALS AND METHODS

1. MATERIALS

1.1. Antibodies

Antibodies were used according to protocols provided by manufacturers. Table 1 summarizes antibodies used in this dissertation research.

Table 1. Antibodies Used

Name	Antigen	Species	Dilution	Manufacturer
H2	KHC	Mouse	1:1000	Developed by Brady Lab
63-90	Dephosphorylated KLC	Mouse	1:500	Developed by Brady Lab
DM1A	a-tubulin	Mouse	1:100K	Sigma Aldrich T9026
R-325	DHC	Rabbit	1:200	Santa Cruz sc-9115
DIC 74-1	DIC	Mouse	1:500	Santa Cruz sc-13524
p150Glued	p150Glued	Mouse	1:250	BD Transduction 610473
PA1-642	Kif5A	Rabbit	1:500	Affinity Bioreagents
UIC81	Kif5B	Rabbit	1:250	Developed by Brady Lab
uKHC	Kif5C	Mouse	1:100	Developed by Brady Lab
Synaptophysin	Synaptophysin	Rabbit	1:250	Zymed 18-0130
GAPDH	GAPDH	Mouse	1:500	Abcam ab9484
GSK3 β	Total GSK3 β	Rabbit	1:1000	BD Transduction lab 610201
Phospho-GSK3 β S9	Inactive GSK3 β	Mouse	1:500	Cell Signaling 9336
Cy5 fluorescent tagged secondary	Mouse IgG	Goat	1:2500	Amersham PA45010
Cy5 fluorescent tagged secondary	Rabbit IgG	Goat	1:2500	Amersham PA45012
HRP-conjugated secondary	Mouse IgG	Goat	1:60K	Jackson Immuno 115-035-146

1.2. Kinases and inhibitors

The following Kinases, Phosphatase, and Kinase inhibitors were used in this dissertation research: CK2 (New England Biolabs P6010 and Millipore #14-197); GSK3b (Sigma Aldrich G-1663); JNK1, JNK2, and JNK3 (Millipore #14-327, #14-329, and #14-501 respectively); Antarctic Phosphatase (New England Biolabs M0289); SAPK inhibitor (Calbiochem SB203580); CK2 inhibitors (Calbiochem DMAT #218699m TBCA #218710); JNK inhibitors (Calbiochem, SP600125 #420119, JIP peptide #420133). Kinase inhibitors were diluted in DMSO. Spermine was purchased from Sigma (S3256), and diluted in water. Peptide substrate for CK2, GSK3b, and background control for GSK3b were synthesized by UIC protein research laboratory. c-Jun, JNK substrate was purchased from Calbiochem (#420108).

1.3. Alzheimer's disease mouse model

Homozygous PS1M146V-ki male mouse was generous gift from Dr. Mattson (NIA). PS1M146V-ki mice were developed by Drs. Guo and Mattson (Guo et al., 1999) by replacing exon 5 of mouse endogenous PS1 gene with h by replacing exon 5 of murine PS1 gene with “humanized” murine PS1 bearing M146V mutation. This animal expresses mutant PS1 at normal physiological levels. Homozygous PS1-ki male was mated with WT C57BL6 female mice to generate heterozygous mice. Pups generated by intercrossing the heterozygous mice were screened for homozygous genotype by PCR followed by restriction enzyme digestion. Mice colonies were maintained at the UIC Biologic Resources Laboratory. All protocols were approved by the Institutional Animal Care and Use Committee at UIC.

1.4. Brain tissue of 5xFAD mouse

Brains of 4months 5xFAD mice (Oakley et al., 2006) were generous gift from Dr. Ladu. Brain was snap frozen in liquid nitrogen right after it was dissected, and kept at -80° until ready to be used.

1.5. Synthetic A β 42 peptide of oligomeric conformation

Oligomeric A β 42 peptide was prepared as described previously (Stine, et al., 2003), and conformation was confirmed using atomic force microscopy.

2. METHODS

2.1. Motility assays in isolated squid axoplasm

Axoplasms extruded from giant axons of squid (*Loligo pealii*; Marine Biological Laboratory) were used for the motility studies as described previously (Brady et al., 1985; Morfini et al., 2006; Szebenyi et al., 2003). Briefly, up to one inch of squid giant axon is dissected, and axoplasm is extruded out onto glass coverslip retaining its morphology and polarity. A chamber is created around the axoplasm with glass coverslips for perfusion of different effectors. Lack of a plasma membrane around the axoplasm allows the study of wide array of molecules such as antibodies (Stenoien and Brady, 1997) and recombinant proteins (Ratner, et al. 1998) on FAT without involvement of membrane permeability and internalization issues. Active kinase, kinase inhibitors, and recombinant proteins were perfused to axoplasm in the Buffer X/2 (175mM potassium aspartate, 65mM taurine, 35mM betaine, 25mM glycine, 10mM HEPES, 6.5mM MgCl₂, 5mM EGTA, 1.5mM CaCl₂, 0.5mM glucose [pH 7.2],

supplemented with 5mM ATP). The velocities of vesicle motility were visualized using video enhanced contrast DIC Zeiss Axiomat microscope (Carl Zeiss, Inc., Thornwood, NY) with a 100x, 1.3NA plan apochromatic objective. Real time image was acquired with Hamamatsu Argus 20 cameras. Instead of measuring the velocity of an individual vesicle which can be difficult due to follow since it can go in and out of the focal plane, the “waves” of vesicles moving in either anterograde or retrograde direction were matched to pre-calibrated cursors and the velocity was recorded. The velocity measurements were taken for 50 minutes, although extruded axoplasms retain baseline velocity for over 2 hours.

2.2. Primary cortical neuron culture

Cortices were dissected from C57BL6 WT or PS1M146V-ki embryos at gestation day 17. Tissues were dissociated in 0.25% Trypsin in 1x Hanks Balanced Salt Solution (Invitrogen) for 17 minutes in 37°C water bath. Cells were counted and plated at 80~100,000 cells/cm² on Poly-L-Lysine (0.1mg/ml) coated plates. Cells were initially plated in MEM media containing 0.6% D-Glucose, 100U/ml Pen-Strep, and 5% fetal bovine serum. After 2~4 hours, culture media was changed to MEM media containing 0.6% D-Glucose, 100U/ml Pen-Strep, N2 and B27 supplements (Invitrogen), and culture was maintained under humidified atmosphere containing 5% CO₂ at 37°C. One-third of culture media was replaced every 3~4days.

2.3. Maintenance and differentiation of N2a cell lines

N2a mouse neuroblastoma cell lines transfected with human wild type PS1 (hPS1 WT) or human PS1 with deletion of exon 9 (hPS1 dE9) (Borchelt, et al., 1996) were generously given by Dr. Lazarov. Cells were grown in media composed of 45% DMEM, 50% Opti-MEM, 5% FBS,

plus 100U/ml Pen-Strep and 2mM L-Glutamine, and maintained under humidified atmosphere containing 5% CO₂ at 37°C. For differentiation, cells were plated at 1000cells/cm², media was changed to differentiation media (49% DMEM, 50% Opti-MEM, 1% FBS, 0.5mM db-cAMP, 100U/ml Pen-Strep, and 2mM L-Glutamine) after 24hours, and differentiated for 5days.

2.4. Immunoblotting

Proteins were separated by electrophoresis in 4-12% Bis-Tris SDS-PAGE gels at 40mA for 2 hours, and transferred onto polyvinylidene fluoride (PVDF) membranes at 0.4A for 2.5 hours. After transfer, membranes were stained with Ponceau to visualize proteins, then cut as needed. Immunoblots were blocked with 5% nonfat dried milk in PBS (pH7.4) for 1 hour at room temperature. Primary antibodies in 1% BSA were added overnight at 4°C with gentle rocking. Membranes were washed 3x 10 minutes with PBST (0.1% Tween), and secondary antibodies in blocking buffer were added for 1 hour at 4°C with gentle rocking. Membranes were again washed 3x 10 minutes before visualized by enhanced chemiluminescence (Amersham) or fluorescence using Typhoon scanner (GE) for quantification. Imagequant software (GE) was used to quantify the band intensity.

2.5. Immunoprecipitation of murine Kinesin-1

Mouse brain was homogenized in homogenization buffer (10mM HEPES, pH 7.4, 150mM NaCl, 1% Triton X-100, 1/100 protease inhibitor cocktail for mammalian tissue (Sigma Aldrich), 1μM PKI peptide (New England Biolabs), 50nM Okadaic acid, 50nM Microcystin). Lysate was centrifuged at 12,500g(max) for 15minutes at 4°C followed by another centrifugation at 100,000g(max) for 5 minutes at 4°C. The resulting clarified lysate was precleared with Protein

G agarose beads and normal mouse IgG sepharose beads. Precleared lysate was then incubated with H2 (KHC) and Protein G beads overnight at 4°C using rotary tube mixer. Background control incubations with normal mouse IgG beads or Protein G beads alone were also done. The next day, beads were washed with the wash buffer (10mM HEPES, pH7.4, 500mM NaCl, 0.5% Triton X-100, 1mM EDTA, 0.02% Sodium Azide) few times, and resuspended in sample buffer for immunoblot analysis or in appropriate kinase/phosphatase buffer for in vitro phosphorylation. Samples were boiled for 5 minutes before loaded onto SDS-PAGE gel.

2.6. In vitro phosphorylation of Kinesin-1

Immunoprecipitated murine Kinesin-1 was equilibrated in appropriate buffer before it was either phosphorylated by CK2 (500U) or first dephosphorylated by Antarctic Phosphatase (5U) and then phosphorylated by CK2. Phosphorylation reaction was started with addition of γ ATP (MP Biomedicals). Both phosphorylation and dephosphorylation reactions were carried out for one hour each. At the end of one hour, reactions were stopped by addition of sample buffer. Immunoprecipitates were separated in SDS-PAGE gels, stained with coomassie blue to visualize the proteins, dried, and exposed to phosphorimager screen for analysis. Signal intensities of corresponding bands were measured using Imagequant.

2.7. In vitro kinase activity assays

Activities of recombinant CK2, GSK3 β , JNKs, or tissue lysate were measured by 32 P incorporation onto their specific substrates, RRRADDSDDDDD, KRREILSRRPpSYR, and c-Jun peptide, respectively. Kinase reactions were started by the addition of γ ATP (MT Biomedicals). For CK2 and GSK3 β , at predetermined time points, aliquots were spotted onto

P81 phosphocellulose paper (Whatman), and rinsed with 75mM orthophosphoric acid four times for 10 minutes each to remove unbound phosphate. The radioactivities were counted in scintillation counter (Beckman Coulter). For JNK activity, aliquots were taken at predetermined time points, and sample buffer was added to stop the further kinase reaction. Samples were then separated in SDS-PAGE gel, stained with Coomassie blue to visualize the proteins, dried, and exposed to phosphorimager screen for analysis. Signal intensities of corresponding bands were measured using Imagequant.

2.8. Kinase activity assays with permeabilized primary mouse cortical neurons

Primary mouse cortical neuron cultures were prepared as described above. Neurons were plated in 24-well plate (BD Falcon) at 200K cells per well. Kinase activity assays were performed on slide warmer set at 37°C located in the area designated for work with radioactive materials. Media was removed from each well, and cultured neurons were rinsed with pre-warmed extracellular buffer (30mM HEPES, pH7.2, 110mM NaCl, 10mM KCl, 1mM MgCl₂, 1.5mM CaCl₂, 10mM Glucose) briefly. Reaction was started by replacing extracellular buffer with 300μl of permeabilization buffer (5mM HEPES, pH7.4, 120mM KCl, 10mM MgCl₂, 1mM KH₂PO₄, 1mM EGTA, 300μM CaCl₂) containing 1.5U Streptolysin-O (Sigma), 15μCi γATP, appropriate kinase substrate peptides, and activators and inhibitors as needed. Streptolysin-O is a hemolytic streptococcal exotoxin that permeabilizes cells by creating holes in cholesterol-enriched plasma membrane which will allow molecules of up to 100KDa to enter the cell. At 10, 20, 30, 40, and 50 minutes, 20μl of the permeabilization mix was spotted onto P81 phosphocellulose paper, and activities were counted in scintillation counter.

2.9. Purification of porcine tubulin

Porcine brains were obtained from UIC BRL through tissue sharing program. Generally, the brains were removed within few hours after animals were sacrificed. Purification of tubulin through two cycles of polymerization and depolymerization was performed according to the protocol developed by Castoldi and Popov (2003). Briefly, dissected porcine brains void of meninges and blood clots were homogenized in 1ml of Depolymerization buffer (50mM MES, pH6.6, 1mM CaCl₂) per gram of tissue. The homogenate was centrifuged to remove large debris, and the supernatant was mixed with High-molarity PIPES buffer (1M PIPES, pH6.9, 10mM MgCl₂, 20mM EGTA), Glycerol, ATP, and GTP. ATP promotes removal of ATP dependent contaminating proteins such as molecular motors. The mixture was incubated at 37°C, and polymerized tubulin was pelleted by centrifugation. Tubulin was then depolymerized in DB at 4°C. This cycle of polymerization and depolymerization was repeated one more time to ensure as much as contaminating proteins were removed. Tubulin was resuspended in BRB80 buffer (80mM PIPES, pH6.8, 1mM MgCl₂, 1mM EGTA) after second polymerization. The final depolymerized tubulin was snap frozen in liquid nitrogen in small aliquots until ready to use. The ability to polymerize and the purity of purified tubulin were assessed and confirmed with SDS-PAGE gel electrophoresis and Coomassie staining.

2.10. Polymerization of purified porcine tubulin

Approximately 700µg of purified tubulin was diluted in 1ml of BRB80 buffer, which results in final tubulin concentration of 7µM, containing 20µM Taxol (Sigma), and 0.1mM GTP. The mixture was incubated for 30 minutes at 37°C before centrifuged for 15 minutes at 50,000RPM

in Optima TL centrifuge at 37 °C to pellet polymerized tubulin. The pellet was resuspended in 100µl of HEPES80 buffer for further processing in microtubule binding assay.

2.11. Microtubule binding assay

Microtubule binding assays were done as described before (Morfini, et al., 2006) with slight modifications. Cells were scraped in HEPES80 (80mM HEPES, pH6.8, 1mM MgCl₂, 1mM EGTA, 1µM Staurosporine, 50nM Okadaic Acid, 200nM Microcystin, and 1/100 protease inhibitor cocktail for mammalian tissue), and homogenized by passing the lysate through 27G needle attached to a syringe three times and through 30G needle twice. The lysate was centrifuged first at 14,000RPM in tabletop centrifuge for 15 minutes at 4°C and then at 55,000RPM in Optima TL centrifuge (Beckman) for 5 minutes at 4°C. The clarified lysate was adjusted to 10mM MgCl₂, and incubated with 0.5mM AMP-PNP (Sigma), polymerized porcine tubulin, 20µM Taxol, and appropriate effector for 30 minutes at 37°C. The mixture was then loaded onto 20% sucrose cushion and centrifuged at 50,000RPM for 20 minutes at 37 °C. The resulting supernatant and microtubule enriched pellet were analyzed with immunoblotting for Kinesin and Dynein.

2.12. Iodixanol vesicle flotation assay

Cells were scraped in homogenization buffer (10mM HEPES, pH7.4, 1mM EDTA, 0.25M sucrose, 1/100 protease inhibitor cocktail for mammalian tissue, 1/100 phosphatase inhibitor cocktail, 2µM K252a, and 1µM PKI), and homogenized by three passages through a 27G needle and two passages through a 30G needle attached to a syringe. The lysate was centrifuged at 100g(max) for 5 minutes at 4°C to remove un-homogenized cells, and combined

with 200 μ l:300 μ l lysate to solution D (50% (w/v) Iodixanol (Sigma), 10mM MgCl₂, 0.25M sucrose). A 500 μ l layer of solution E (25% (w/v) Iodixanol, 10mM MgCl₂, 0.25M sucrose) was gently loaded on top of the lysate adjusted to 30% Iodixanol, followed by a 400 μ l layer of solution F (5% (w/v) Iodixanol, 10mM MgCl₂, 0.25M sucrose). Samples are centrifuged at 250Kg(max) for 80 minutes at 4°C in TLS-55 swing bucket rotor in Optima TL centrifuge. Following the centrifugation, 200 μ l was removed from top and discarded. Fractions 150 μ l were collected from liquid-air interface the first fraction was labeled as fraction 1, for the total of 8 fractions. Samples were analyzed by immunoblotting.

2.13. Tissue lysate preparation for developmental study

Cortex, hippocampus, and cerebellum were dissected from wild type C57BL6 mice at 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28 days, 3, 6, 9, 19 months of age. The tissues were homogenized in 10mM HEPES, pH 7.4, 150mM NaCl, 1% Triton X-100, 1/100 protease inhibitor cocktail for mammalian tissue. Homogenates were then centrifuged at 14,000RPM for 15 minutes at 4°C tabletop centrifuge. Supernatant was subjected for another centrifugation. The protein concentration of the clarified lysate was measured using BCA protein assay kit (Pierce). Samples were analyzed by immunoblot for relative levels of different motor protein components with GAPDH used as loading control.

CHAPTER IV

RESULTS

1. To evaluate the effect of oligomeric A β 42 on fast axonal transport.

1.1. Active CK2 inhibits both directions of fast axonal transport in squid axoplasm.

Axonal transport is regulated by activities of phosphotransferases, and several kinases and phosphatases have been shown to directly or indirectly affect FAT. For example, JNK inhibits FAT in both directions (Morfini et al., 2006), GSK3 β inhibits anterograde and not retrograde FAT (Morfini et al., 2002b), and the catalytic subunit of PKC increase retrograde transport while minimally affecting anterograde transport (Morfini et al., 2007). To examine the effect of the serine/threonine kinase CK2, we perfused 4 units of recombinant human CK2 into isolated squid axoplasm. CK2 is abundantly expressed in mammalian brain (Guerra et al., 1999) and shown to be dysregulated in Alzheimer's disease (Masliah et al., 1992). Velocity measurements were taken for a period of 50 minutes, although squid axoplasms retain vesicle motilities for several hours after extrusion (Brady et al., 1985). In buffer control conditions, the velocities of anterograde transport remain at around 1.7 μ m/sec and velocities of retrograde remain at around 1.3 μ m/sec for the duration of measurement (Figure 1A). However, both directions of transport were significantly impaired when active CK2 was perfused (Figure 1B). The effects of CK2 on FAT were seen within a few minutes after kinase perfusion. Like aforementioned kinases, CK2 inhibits both directions of axonal transport.

Figure 1

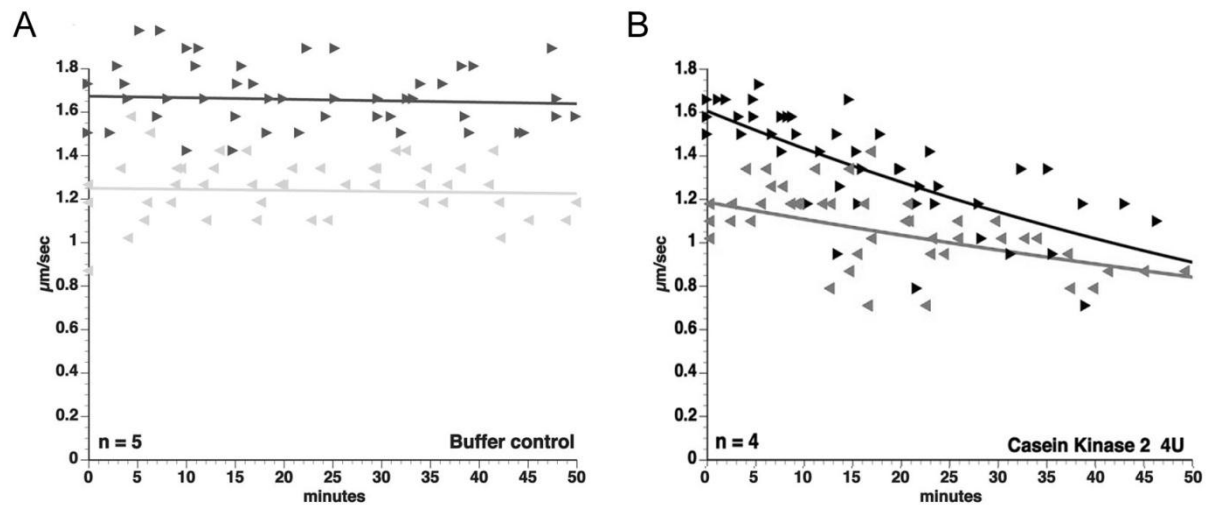


Figure 1. Active CK2 inhibits fast axonal transport.

Isolated squid axoplasms were perfused with buffer X alone (A) or with active CK2 in buffer X (B) to determine effects of CK2 on FAT. Each black arrow represents an individual anterograde transport velocity measurement, and each gray arrow represents an individual retrograde transport velocity measurement. Measurements were made for 50 minutes. "n=" in each plot indicates the number of axons used for each conditions. Perfusion of active CK2 (4U) significantly inhibited both anterograde and retrograde FAT.

1.2. CK2 mediates inhibition of fast axonal transport by oligomeric A β 42.

Next, we proceeded to assess the effect of oligomeric A β 42 (oA β 42) on FAT. A β 42 is one of the main components of amyloid plaques, a pathological hallmark of AD. A β 42 can form oligomers and fibrillar aggregates, and in recent years, oligomeric forms of A β 42 have been shown to be more neurotoxic. Perfusion of oA β 42 into the squid axoplasm significantly inhibited both directions of FAT (Figure 2A). The pattern of FAT inhibition by oA β 42 is very similar to inhibition of FAT by JNK and CK2. Furthermore, concentration of oA β 42 perfused (100nM) is far less than the concentration of kinesin-1 (500nM) (Pfister et al., 1989), the anterograde motor protein. This suggests oA β 42 is triggering activation of an enzymatic cascade rather than physically sequestering the motor proteins themselves. To examine which phosphotransferase is activated by oA β 42, SAPK and CK2 inhibitors were co-perfused with oA β 42. Of note, perfusion of kinase inhibitors alone does not affect FAT. The SAPK inhibitor (SB203580) did not prevent the inhibition of FAT (Figure 2B), but a highly specific CK2 inhibitor, TBCA, completely blocked the effect of oA β 42 on FAT (Figure 2C). Similarly, perfusion of a CK2 specific peptide substrate to divert the kinase activity fully prevented the FAT inhibition by oA β 42. This confirms that activation of endogenous CK2 mediates inhibition of FAT in squid axoplasm by oA β 42.

1.3. Oligomeric A β 42 increase phosphorylation of Kinesin light chains, and activate CK2.

To confirm that perfusion of oA β 42 leads to increases in the kinase activity of CK2, lysate prepared from squid axoplasms perfused either with oA β 42 or with monomeric,

Figure 2

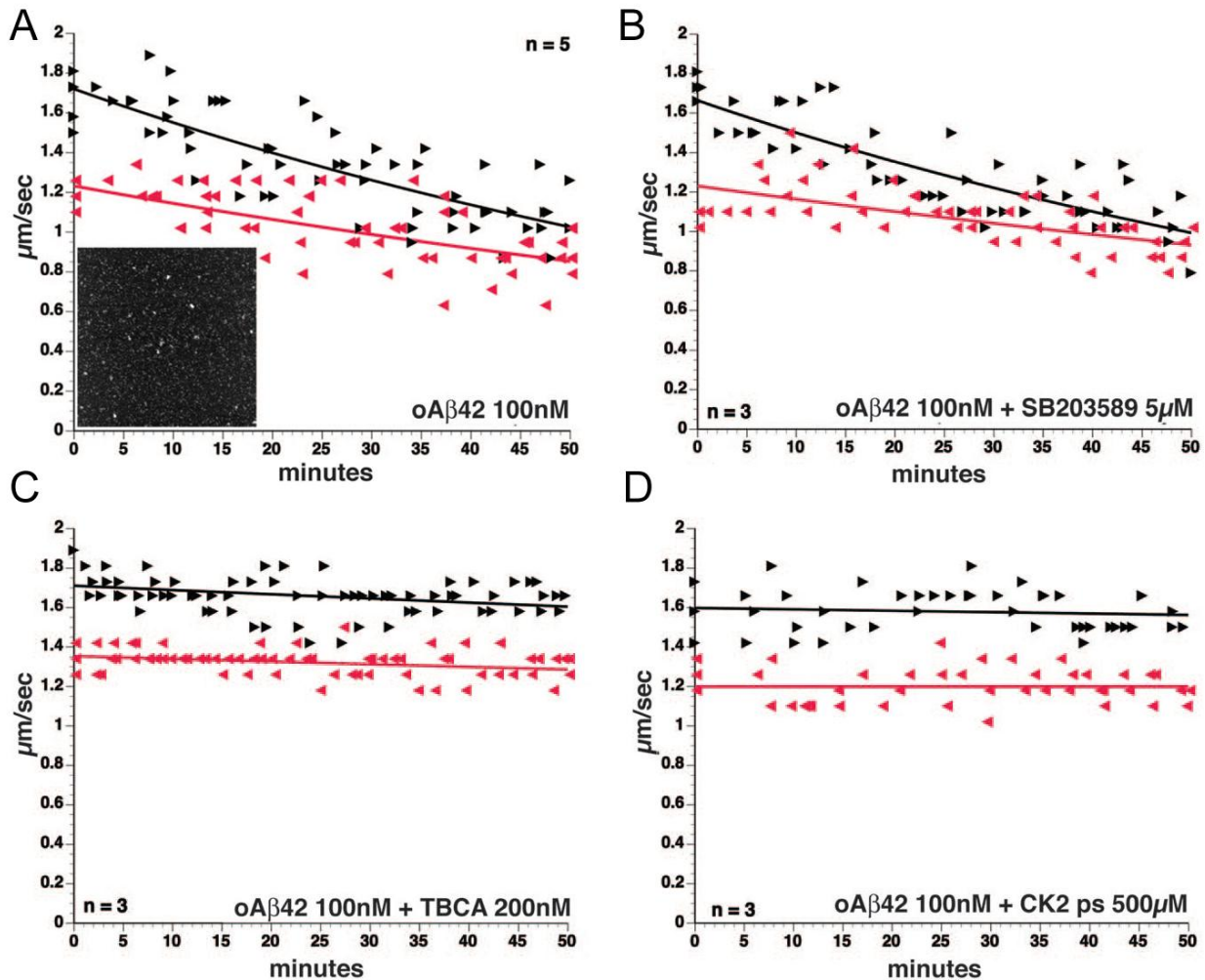


Figure 2. CK2 mediates inhibition of fast axonal transport by oligomeric A β 42.

100nM of oligomeric A β 42 was perfused in isolated squid axoplasms with or without various kinase inhibitors. Each black arrow represents an individual anterograde transport velocity measurement, and each gray arrow represents an individual retrograde transport velocity measurement. Measurements were made for 50 minutes. "n=" in each plot indicates the number of axons used for each condition. (A) Perfusion of oA β 42 significantly impaired both directions of FAT. (Insert in A) Atomic force microscopy image of oA β 42. (B) Co-perfusion of SAPK inhibitor SB203589 did not prevent the effect of oA β 42. (C) Co-perfusion of CK2 inhibitor TBCA or (D) peptide substrate of CK2 fully prevented the inhibition of FAT caused by oA β 42.

Figure 3

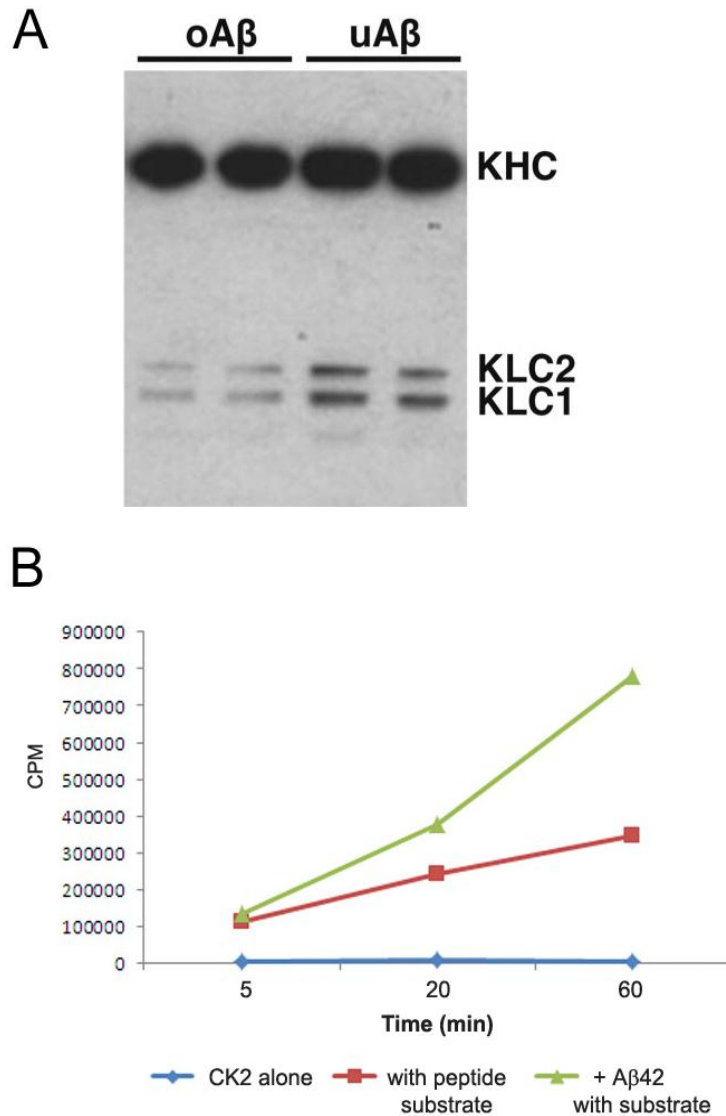


Figure 3. Oligomeric Aβ42 increases kinesin light chain phosphorylation and CK2 activity.

(A) Immunoblot of squid axoplasm perfused with oAβ42 or monomeric uAβ42. Squid axoplasm lysate was analyzed by H2 (KHC), loading control, and 63-90 (KLC) antibodies. 63-90 preferentially recognizes dephosphorylated KLCs. The reduced immunoreactivity of KLCs in oAβ42 perfused samples suggests that perfusion of oAβ42 leads to an increase in squid KLCs phosphorylation by endogenous kinase. (B) In vitro kinase assays with recombinant CK2. Co-incubation of oAβ42 with recombinant CK2 increases ³²P incorporation into the peptide substrates compared to CK2 alone. oAβ42 activates CK2 and increases phosphorylation of KLCs.

unaggregated A β 42 (uA β 42), which has no effect on FAT (data not shown), were analyzed with 63-90, an antibody against KLC (Figure 3A). 63-90 preferentially recognizes dephosphorylated KLCs (Pigino et al., 2003). Using KHC as loading control, immunoreactivity of 63-90 in oA β 42 treated axoplasm is significantly reduced, indicating that increased phosphorylation on KLC. In vitro kinase activity assay of CK2 was performed to see if oA β 42 can directly activate CK2 (Figure 3B). After 60minues of incubation, the amount of radiolabeled phosphate incorporation onto CK2 specific peptide substrate in CK2 + A β 42 condition is 2 to 2.5 time higher than that of CK2 and substrate without A β 42. CK2 alone without peptide substrate served as background control which is negligible. Both immunoblot and kinase activity assay demonstrate that oA β 42 indeed activates CK2 which increase the phosphorylation of anterograde motor, kinesin-1.

2. To investigate the molecular mechanism of impairment of fast axonal transport by CK2 activation.

2.1. CK2 phosphorylates both heavy and light chains of kinesin-1.

Regulation of FAT by phosphotransferases involves direct or indirect phosphorylation of kinesin. Cyclin dependent kinase 5 (cdk5), for example, prevents impairment of FAT by suppressing the activity of PP1 that dephosphorylates GSK3 β , resulting in activation of GSK3 β which in turn phosphorylates KLC and inhibit anterograde transport (Morfini et al., 2004). To investigate if CK2 directly phosphorylates Kinesin, immunoprecipitated murine kinesin-1 was phosphorylated with recombinant human CK2 holoenzyme with or without CK2 inhibitor, DMAT (Figure 4A). Both heavy (KHC) and light chains (KLC1 and KLC2) are readily

Figure 4

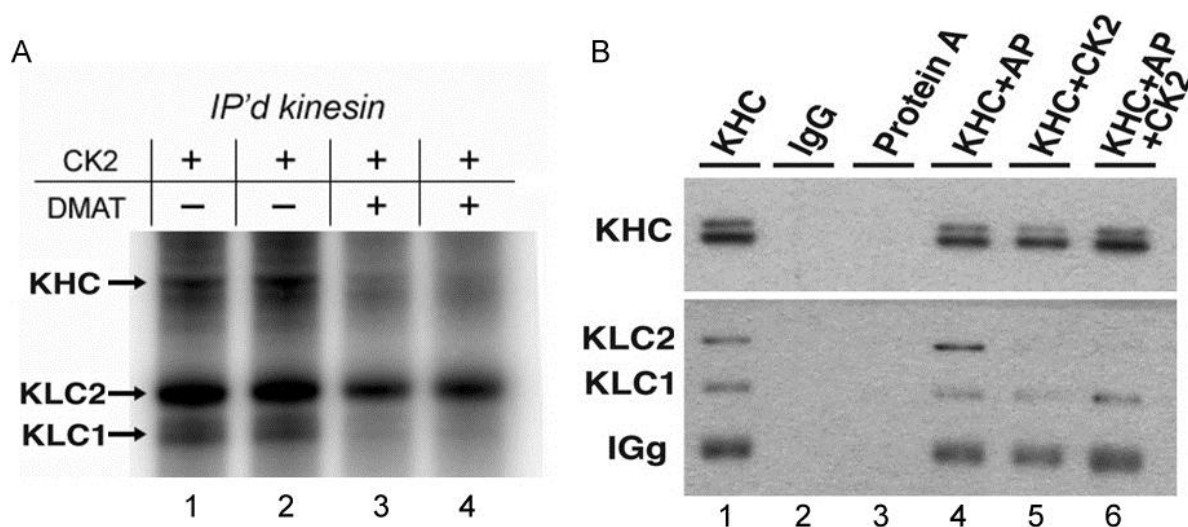


Figure 4. CK2 phosphorylates both heavy and light chains of kinesin.

(A) Immunoprecipitated (IP) mouse Kinesin-1 was phosphorylated by recombinant CK2 with or without the inhibitor DMAT. Kinesin-1 was immunoprecipitated from murine brain using H2 antibody. Autoradiogram showing incorporation of ^{32}P into IP KHC and KLCs when incubated with recombinant CK2 (lanes 1 and 2), and reduced ^{32}P incorporation when co-incubated with the CK2 inhibitor, DMAT (lanes 3 and 4). (B) Immunoblot analysis of Kinesin-1 IP. Kinesin-1 was immunoprecipitated from murine brain using H2 and analyzed by H2 (KHC) and 63-90 (KLCs) antibodies. Note 63-90 immunoreactivity increased when the Kinesin-1 IP was dephosphorylated by Antarctic phosphatase (AP) (lane 4). Conversely, 63-90 immunoreactivity decreased when IP Kinesin-1 was phosphorylated by CK2 (lane 5) or when phosphorylated by CK2 after dephosphorylated by AP (lane 6). Control immunoprecipitates with normal murine IgG (lane 2) and protein A agarose beads (lane 3) are also shown.

phosphorylated by CK2 (Figure 4A, lanes 1&2), and radiolabeled phosphate incorporation decreases in presence of CK2 inhibitor (Figure 4A, lanes 3&4). The phosphorylation state sensitive antibody against KLC, 63-90, was raised against bovine brain kinesin and the epitope on murine KLCs has been mapped to the first 50 amino acids (Stenoien and Brady, 1997). One of the consensus sites for CK2 phosphorylation is within the first 50 amino acids. To examine if 63-90 is CK2 dependent antibody, we characterized the antibody with immunoprecipitated (IP) murine Kinesin-1. Samples were also blotted with H2 (KHC) to show even protein loading. Both KLC1 and KLC2 were recognized in immunoprecipitates from murine brain (Figure 4B, lane 1). Background control pull-downs with normal mouse IgG agarose beads and Protein A sepharose beads do not contain Kinesin (Figure 4B, lanes 2 and 3, respectively), and this confirms the specificity of IP using H2 antibody. Kinesin is a phosphoprotein (Hollenbeck, 1993), and dephosphorylating the Kinesin with Antarctic Phosphatase (AP) increased immunoreactivity of KLCs (Figure 4B, lane 4) further demonstrating that 63-90 preferentially recognizes dephosphorylated KLCs. When Kinesin was phosphorylated by CK2 or dephosphorylated by AP prior to phosphorylation by CK2, immunoreactivity of KLCs dramatically decreased (Figure 4B, lanes 5 and 6, respectively). This is consistent with the idea that 63-90 preferentially recognizes KLCs that are not phosphorylated at CK2 epitope.

2.2. CK2 phosphorylates kinesin heavy chain at Serine 175.

To further investigate molecular mechanisms of FAT impairment caused by CK2 activation, Serine at 175 in KHC has been identified as potentially phosphorylated by CK2, and a former graduate student Yimei You generated three recombinant truncated forms of KHC which include microtubule (MT) binding/ATPase and part of coiled-coil neck + hinge domains,

Figure 5

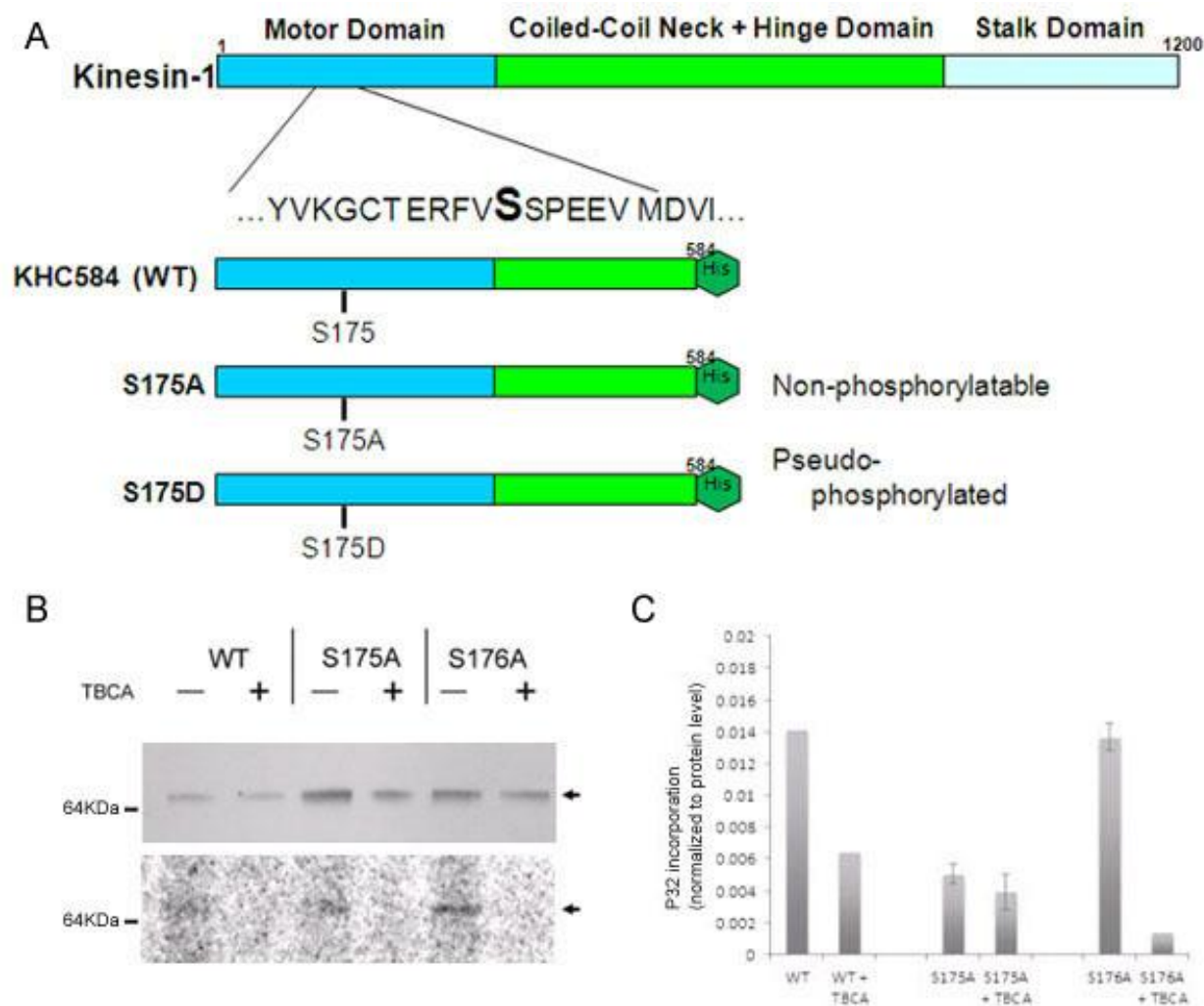


Figure 5. CK2 phosphorylates kinesin heavy chain at Serine 175.

(A) Diagram of full length Kinesin-1 and different KHC584 constructs. Top: Diagram of full length Kinesin-1 protein. A previous study had identified Serine175 as the CK2 epitope. Bottom: Three constructs of truncated Kinesin-1 (KHC584) were generated, expressed, and purified from bacterial expression system. Serine175 was mutated into Alanine (S175A) to generate a non-phosphorylatable form. Also, Serine175 was pseudophosphorylated by mutating it to Aspartic Acid (S175D). A control KHC584 construct which Serine at 176 is mutated into Alanine (S176A) was also used. (B) In vitro phosphorylation of KHC584 by CK2. Top: Immunoblot analysis using H2 antibody to show total protein levels. Bottom: Autoradiogram showing incorporation of ³²P into KHC584 constructs. KHC584 constructs were phosphorylated by CK2 in the absence or presence of CK2 inhibitor TBCA. (C) Quantification of ³²P incorporation into KHC584 constructs. The signal from the autoradiogram was normalized to protein levels from the immunoblot. The ³²P incorporation was lower for S175A construct compared to WT and S176A. ($n=2\sim3$)

KHC584 WT, KHC584 S175A, and KHC584 S175D with point-directed mutagenesis (Figure 5A). Serine175 is replaced with Alanine (KHC584 S175A) to render it non-phosphorylatable, and with Aspartate (KHC584 S175D) to mimic phosphorylated state. All three constructs were incubated with recombinant CK2 in vitro (Figure 5B). The protein loading per construct was confirmed with the immunoblot with H2 antibody (Figure 5B, top panel). Both immunoblot and phosphorimager screen image (Figure 5B, bottom panel) show a single band slightly above 64KDa which corresponds to predicted KHC584 size of 67KDa. The amount of ^{32}P incorporation was calculated by normalizing the signal intensities from phosphorimager screen to the signal from immunoblot. The quantification of in vitro phosphorylation shows ^{32}P incorporation is lower in KHC584 S175A construct compared with two other constructs while addition of TBCA prevents phosphorylation of all three constructs (Figure 5C). This confirms that Serine 175 of KHC is phosphorylated by CK2.

2.3. Mutation on kinesin heavy chain that mimics phosphorylation by CK2 has impaired binding to microtubules.

Phosphorylation of KHC by JNK causes kinesin to be released from MTs and impairs FAT (Morfini et al., 2006). To investigate the effect of KHC phosphorylation by CK2 on MT binding, MT binding assays were performed with KHC584 S175A and KHC584 S175D (Figure 6A). MT binding assay utilizes AMP-PNP, a non-hydrolyzable analogue of ATP, to assess amount of kinesin bound to MT independent of its ATPase activity. In presence of AMP-PNP, kinesin gets “stuck” on MT. However it fails to bind to MT when it is phosphorylated at residue(s) that interfere with its MT binding ability. Tubulin was pre-polymerized with GTP and

Figure 6

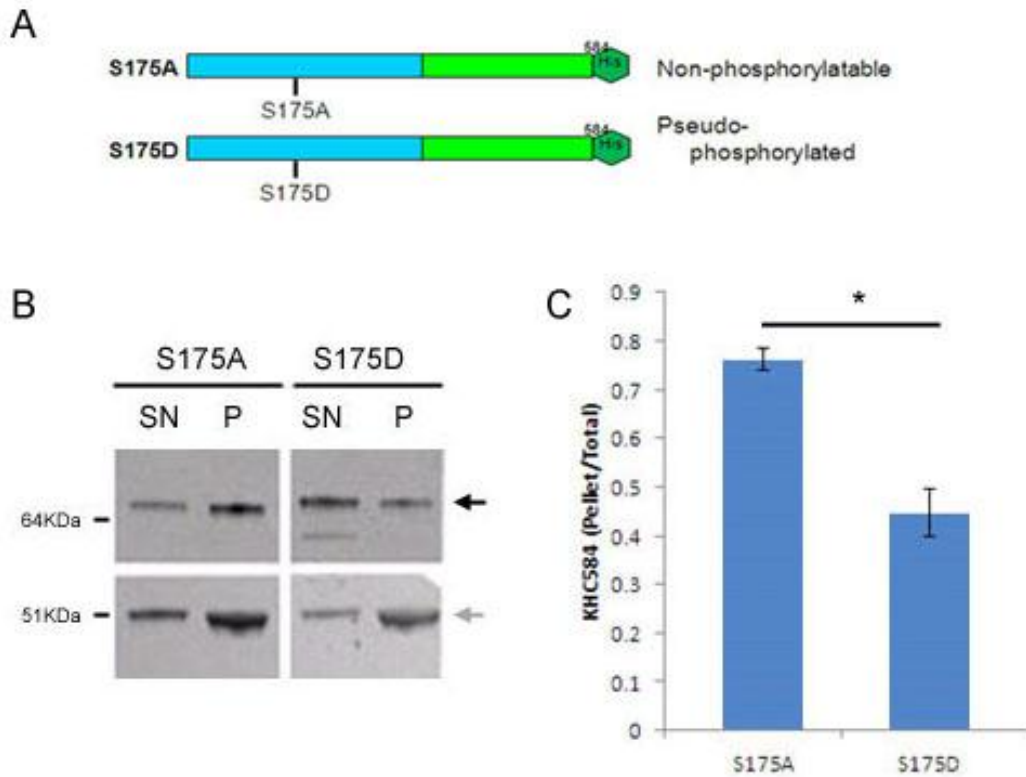


Figure 6. Mutation on the kinesin heavy chain that mimics phosphorylation by CK2 have impaired binding to microtubules. (A) Diagram of two KHC584 constructs used for microtubule (MT) binding study. (B) Immunoblot analysis of MT binding study using H2 (black arrow) and DM1A (gray arrow) antibodies. KHC584 constructs were incubated with purified, pre-assembled porcine tubulin and AMP-PNP, a non-hydrolyzable analogue of ATP. The reaction was centrifuged, and the supernatant (SN) and the microtubule enriched pellet (P) were collected. Note that the tubulin is enriched in the pellet fraction with both constructs indicating successful assay. (C) Quantification of immunoblot. Significantly lower levels of KHC584 in the MT enriched pellet fraction of S175D compared to S175A indicates that the mutation that mimics KHC phosphorylation by CK2 impairs kinesin binding to MT. (* $p < 0.005$, $n = 4$, error bar = \pm SEM, student's t -test)

taxol which promotes MT formation, and enrichment of tubulin in pellet fraction is used to confirm a successful assay. Immunoblot (Figure 6B) and densitometric quantification of the blot (Figure 6C) shows over 70% of KHC584 S175A was attached to the MT, but less than 50% of KHC584 S175D was attached. This indicates that pseudo-phosphorylation of the CK2 modification site on KHC reduces kinesin binding to MT, and may contribute to impairment of FAT.

2.4. Spermine activates CK2 while inhibiting GSK3 β and JNKs.

To further investigate the role of CK2 activation on impaired FAT, we explored specific activator for murine endogenous CK2. Polybasic proteins such as poly-lysine and polyamines are thought to activate CK2 by binding to the acidic region of regulatory CK2 β subunits (Hathaway and Traugh, 1984a; Meggio et al., 1994). As previously reported spermine (SPM), one of the polyamines, significantly increased human recombinant CK2 when incubated together (Figure 7A). CK2 activity assays were done using Streptolysin O (SLO) permeabilized primary mouse cortical neurons (PMCN) to assess the effect of SPM on CK2 activity in a more physiological condition (Figure 7B). The SPM activated mouse endogenous CK2 two to three times compared to without SPM. The effects of SPM on activities of other kinases that are shown to be activated in AD were also evaluated using recombinant kinases. Surprisingly, not only did SPM not activate GSK3 β or JNKs, but it actually inhibited GSK3 β , and all three isoforms of JNKs (Figure 7C, D, and E). Spermine is indeed a potent activator of CK2, and inhibits GSK3 β and JNK.

Figure 7

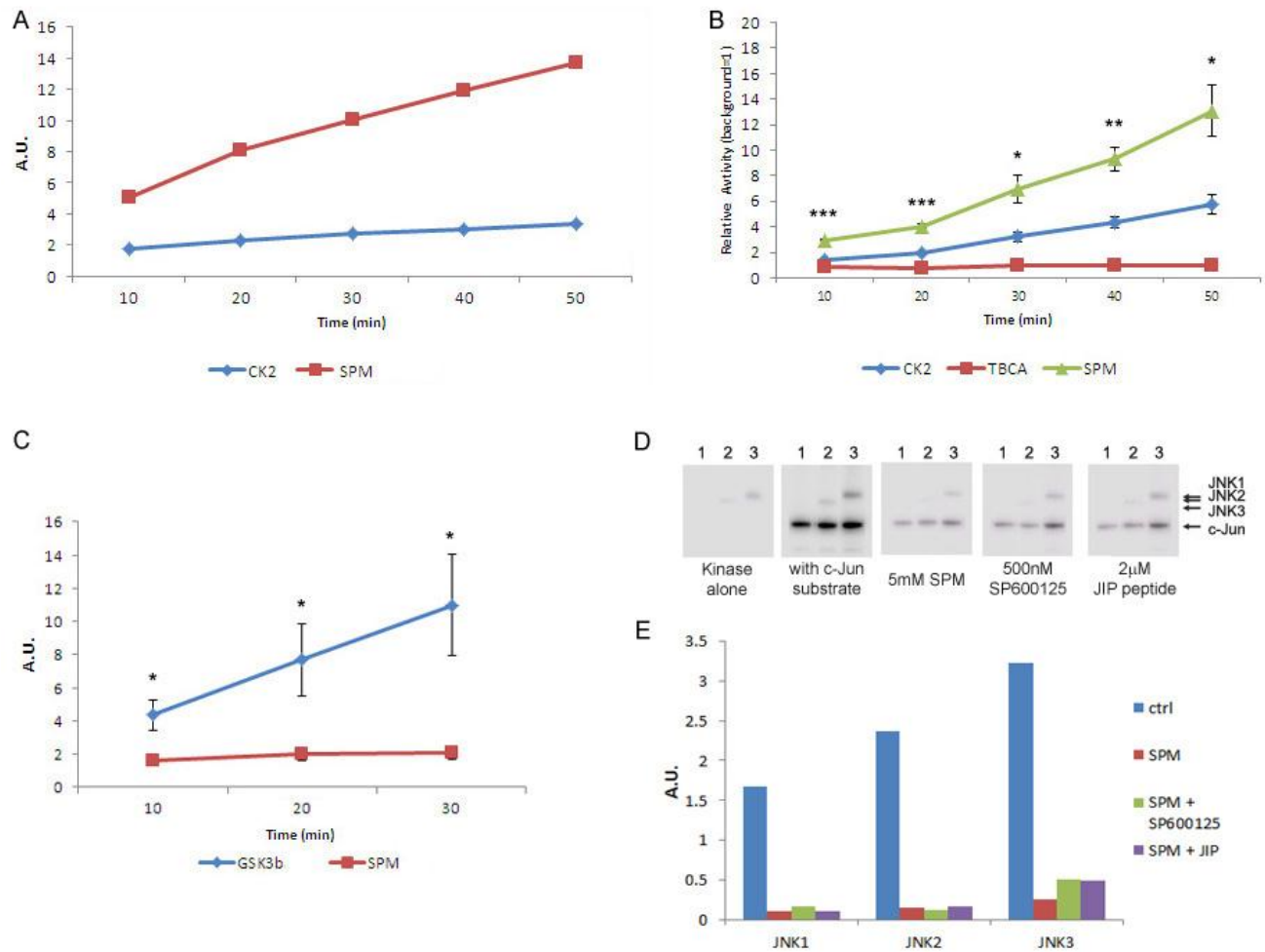


Figure 7. Spermine activates CK2 and inhibits GSK3 β and JNKs.

In vitro kinase activity assay of recombinant CK2 (A) and permeabilized primary mouse cortical neurons at DIV2 (B). Kinase activities were measured with ^{32}P incorporation into CK2 specific peptide substrate. Co-incubation of 5mM spermine significantly increased CK2 activity. (C) In vitro kinase activity assay of recombinant GSK3 β . Kinase activities were measured with ^{32}P incorporation into a GSK3 β specific peptide substrate. Co-incubation with 5mM spermine significantly inhibited GSK3 β activity. (D and E) In vitro kinase activity assay of recombinant JNKs. Kinase activities were measured with ^{32}P incorporation into the JNK substrate protein c-Jun fragment. Both the autoradiogram (D) and the quantification of autoradiogram (E) showed significant inhibition of JNK activities of all isoforms with co-incubation of 5mM spermine. (lane1: JNK1, lane2: JNK2, lane3: JNK3) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$, error bar = $\pm \text{SEM}$, student's t -test)

2.5. Purification of porcine tubulin.

It is imperative in doing MT binding assays, to have high enough concentration of tubulin so that it can polymerize into MT and provide a platform for motor proteins to bind. Critical concentration of tubulin polymerization is around $8\mu\text{M}$ (Na and Timasheff, 1982). In our MT binding assays, taxol which lowers the critical concentration to about $2\mu\text{M}$ (Ganesh et al., 2004) is added to a final concentration of $20\mu\text{M}$ to promote MT formation. However, when MT binding is performed with cell lysate, the proteins in cells are diluted thousands of times as the lysate is collected and tubulin concentration falls far below the critical concentration to polymerize. This can be resolved by adding exogenous tubulin. We have purified porcine tubulin using two cycle polymerization-depolymerization protocol (Castoldi and Popov, 2003)(Figure 8A). Through multiple steps of polymerization and depolymerization, highly pure tubulin is purified at high yield ($700\mu\text{g}$ of tubulin per gram of brain tissue). The entire purification process took about 9 hours instead of the few days that it takes with traditional protocol using a phosphocellulose column. To assess the polymerization properties of purified tubulin prepared this way, polymerization assay with 5 or $10\mu\text{M}$ of tubulin was done in presence of $20\mu\text{M}$ taxol (Figure 8B). At both concentrations, almost all tubulin polymerized and pelleted down after short centrifugation. Using this protocol provided us with very pure and highly active tubulin in a very short amount of time.

Figure 8

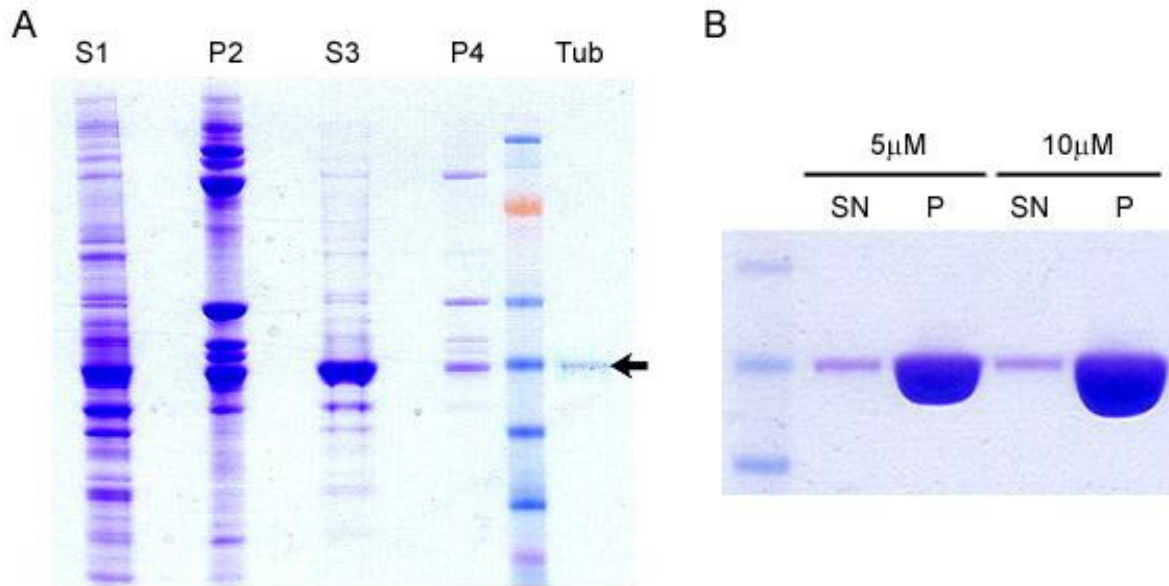


Figure 8. Purification of porcine tubulin.

Tubulin was purified from porcine brain through multiple steps of polymerization and depolymerization (A). Aliquots were collected after the initial spin (S1), the first polymerization (P2), the first depolymerization (S3), and the second polymerization (P4) showing decreasing impurity. Collected fractions were separated in SDS-PAGE gel and stained with coomassie blue. The purified tubulin (tub) shows single band right below the 51KDa molecular marker (black arrow) which corresponds to molecular weight of tubulin, 50KDa. (B) In vitro polymerization assay of purified tubulin. Two different concentrations of purified porcine tubulin were incubated with taxol and GTP to enhance microtubule formation, and centrifuged to separate into a non-polymerized fraction and a polymerized microtubule fraction. Both concentrations of 5μM and 10μM show that most of tubulin was polymerized to form microtubules.

2.6. Activation of mouse endogenous CK2 by spermine reduces kinesin binding to microtubules.

To determine if activation of mouse endogenous CK2 affects kinesin binding to MTs, lysate from primary mouse cortical neuron was pre-incubated with SPM and processed for MT binding assay. Immunoblot analysis of supernatant (SN) and MT enriched pellet (P) (Figure 9A) shows majority of Kinesin stays in SN in ATP ctrl samples (left two lanes) indicating that this experimental condition does not affect ATPase activity of Kinesin. Pre-incubation of lysate with SPM for ATP ctrl did not alter the enrichment of Kinesin in SN fraction either (data not shown). Tubulin immunoblot is included (Figure 9A bottom panel) to show that tubulin successfully polymerized into MT and pelleted down. Using the total KHC antibody, H2, the immunoblot (Figure 9A top panel) and the quantification (Figure 9B) reveals significant reduction in KHC in P fraction when the lysate was pre-incubated with SPM compared to AMP-PNP control. Mouse kinesin has three isoforms, Kif5A, Kif5B, and Kif5C (Xia et al., 1998), and each isoform has different rate of transport thus contributing to specialization of cargoes they carry (Elluru et al., 1995). To determine if different isoforms are affected by activation of CK2 by SPM, immunoblots were analyzed using isoform specific antibodies (Figure 9A middle three panels) and signal intensities were quantified (Figure 9C, 9D, and 9E). Bindings of all three kinesin heavy chain isoforms are significantly reduced by pre-incubation with SPM. Immunoprecipitated kinesin from lysates incubated with γ ATP and SPM (Figure 9F) shows that kinesin phosphorylation is increased in presence of SPM. These data suggest that activation of mouse endogenous CK2 by SPM increase kinesin phosphorylation and reduces its binding to MT.

Figure 9

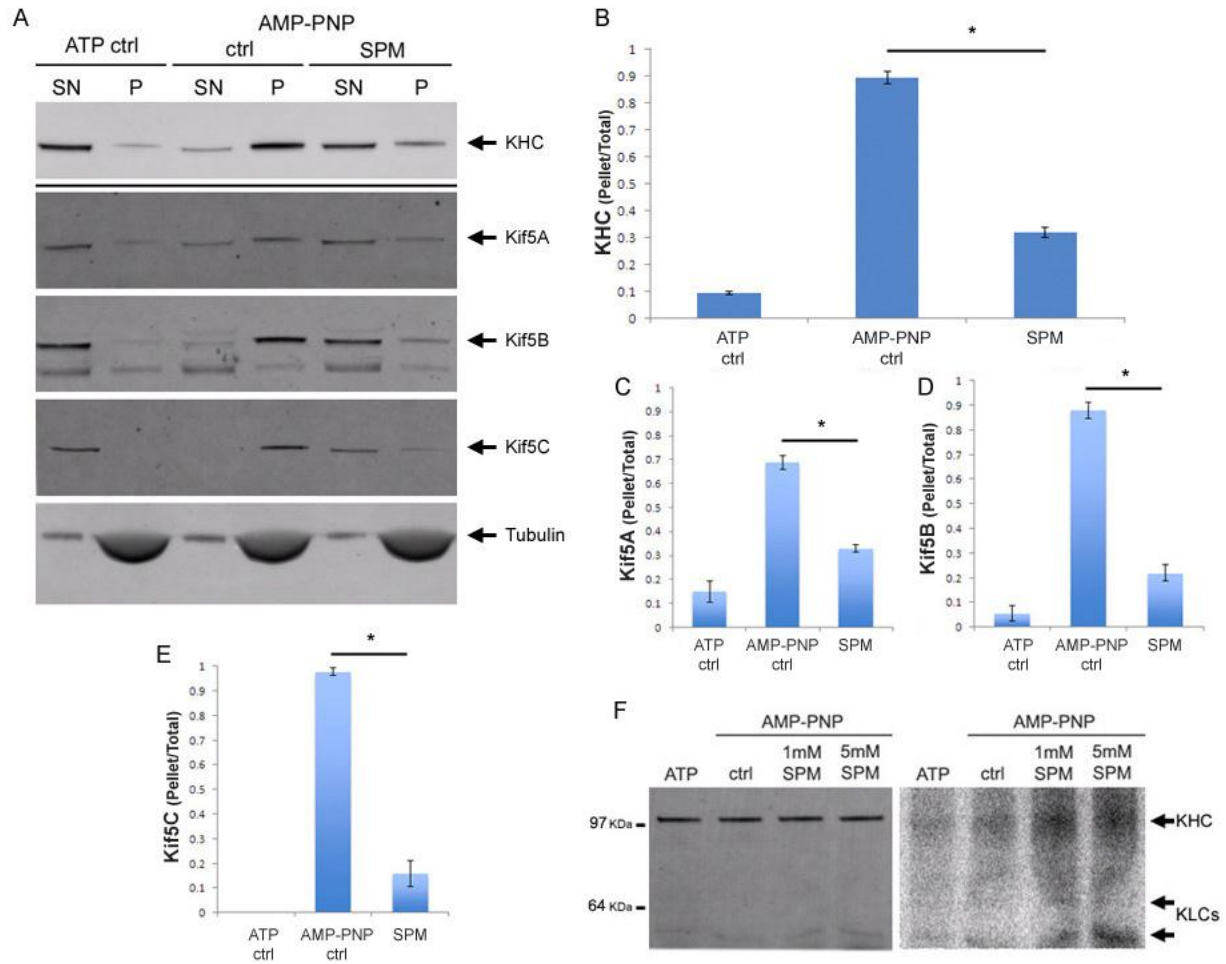


Figure 9. Activation of mouse endogenous CK2 by spermine reduces kinesin binding to microtubules.

Microtubule pellets (P) and supernatant (SN) fractions were prepared from primary mouse cortical neurons at DIV5. **(A)** Immunoblot analysis of microtubule pellets incubated with ATP, AMP-PNP, and AMP-PNP with 5mM spermine. Quantification of kinesin ratio (MT enriched pellet/Total) for total kinesin **(B)**, Kif5A **(C)**, Kif5B **(D)**, and Kif5C **(E)**. Enrichment of tubulin in the pellet fraction confirms successful assay. ATP released almost all Kinesin into the supernatant serving as a control for kinesin's ATPase activity. AMP-PNP, a non-hydrolyzable analogue of ATP, makes kinesin "stuck" on the microtubules, however in all three isoforms of kinesin a significant portion of kinesin was released into the supernatant when mouse endogenous CK2 was activated by co-incubation of spermine. **(F)** Spermine increased kinesin phosphorylation. Cell lysates from primary mouse cortical neurons were treated in the same way as in the microtubule binding assay along with γ ATP. Instead of centrifugation to isolate the MT enriched pellet, kinesin was immunoprecipitated using H2 (KHC) antibody. Immunoblot analysis (left panel) shows equal sample loading. Autoradiogram (right panel) shows that kinesin from samples incubated with spermine is more phosphorylated. (* $p < 0.0001$, $n = 5$, error bar = \pm SEM, student's *t*-test)

2.7. AMP-PNP inhibits CK2

AMP-PNP inhibits kinesin motility by binding tightly to nucleotide binding region of KHC (Uemura et al., 2002), but as an ATP analogue it is also an inhibitor for many other ATP-dependent molecules such as PKC (Leventhal and Bertics, 1991) and single ATP-sensitive potassium channels (KATP channels) (Hehl and Neumcke, 1994). Kinase activity assay was done to examine the inhibitory effect of AMP-PNP on CK2 (Figure 10). As shown before, SPM activates CK2, and specific inhibitor TBCA inhibits CK2. Addition of AMP-PNP inhibited CK2 activity. Addition of SPM in presence of AMP-PNP increased CK2 activity although it was still lower than CK2 alone. This result indicates that AMP-PNP is a potent inhibitor of CK2.

2.8. Effect of spermine on kinesin binding to microtubules is negatively correlated with AMP-PNP concentration.

Using AMP-PNP as an inhibitor of CK2, the effects of SPM on MT binding of kinesin were evaluated with MT binding assay (Figure 11A and B). In all of the conditions used tubulin polymerized and pelleted in the MT enriched pellet. In absence of SPM, over 95% of kinesin is in MT enriched pellet regardless of AMP-PNP concentration (Figure 11A left panel). However, when the lysate was pre-incubated with SPM to activate mouse endogenous CK2 the amount of kinesin in MT enriched panel was significantly lower, and this effect was dependent on AMP-PNP concentration (Figure 11A right panel). When 0.5mM of AMP-PNP was used, there was about a 50% reduction in amount of kinesin in MT enriched pellet with the addition of SPM, but with 2.5mM AMP-PNP the reduction was just around 20% (Figure 11B). To determine if this AMP-PNP concentration dependent effect of SPM was true for all three isoforms of KHCs,

Figure 10

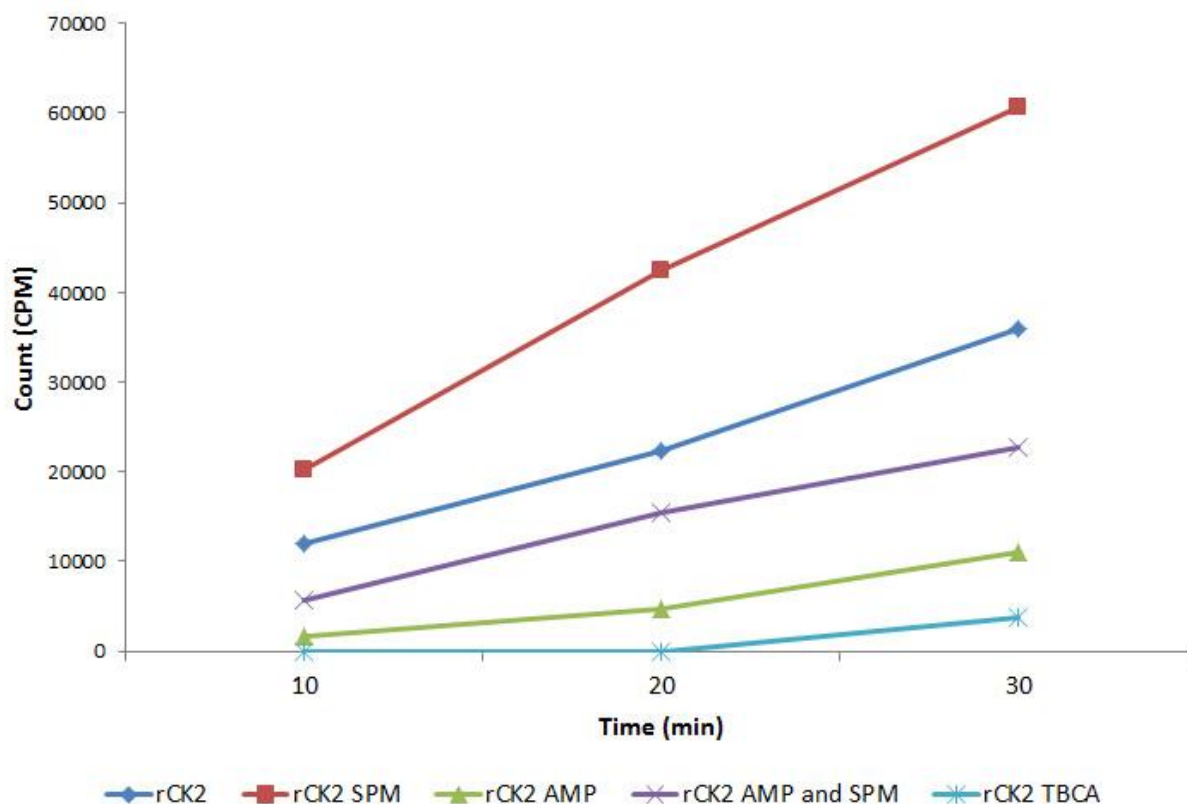


Figure 10. AMP-PNP inhibits CK2.

An in vitro kinase activity assay with recombinant CK2 was performed to see the effect of AMP-PNP, a non-hydrolyzable analogue of ATP, on CK2 activity. As shown in Figure 7, spermine activated CK2 (red line), and the CK2 inhibitor TBCA almost completely suppressed ^{32}P incorporation into the peptide substrate (light blue line) confirming the specificity of the assay. Addition of AMP-PNP to spermine inhibited CK2 activity (purple line) compared to spermine alone, and moderately compared to the CK2 control condition (dark blue line). Addition of AMP-PNP alone to CK2 further inhibited CK2 activity (green line).

Figure 11

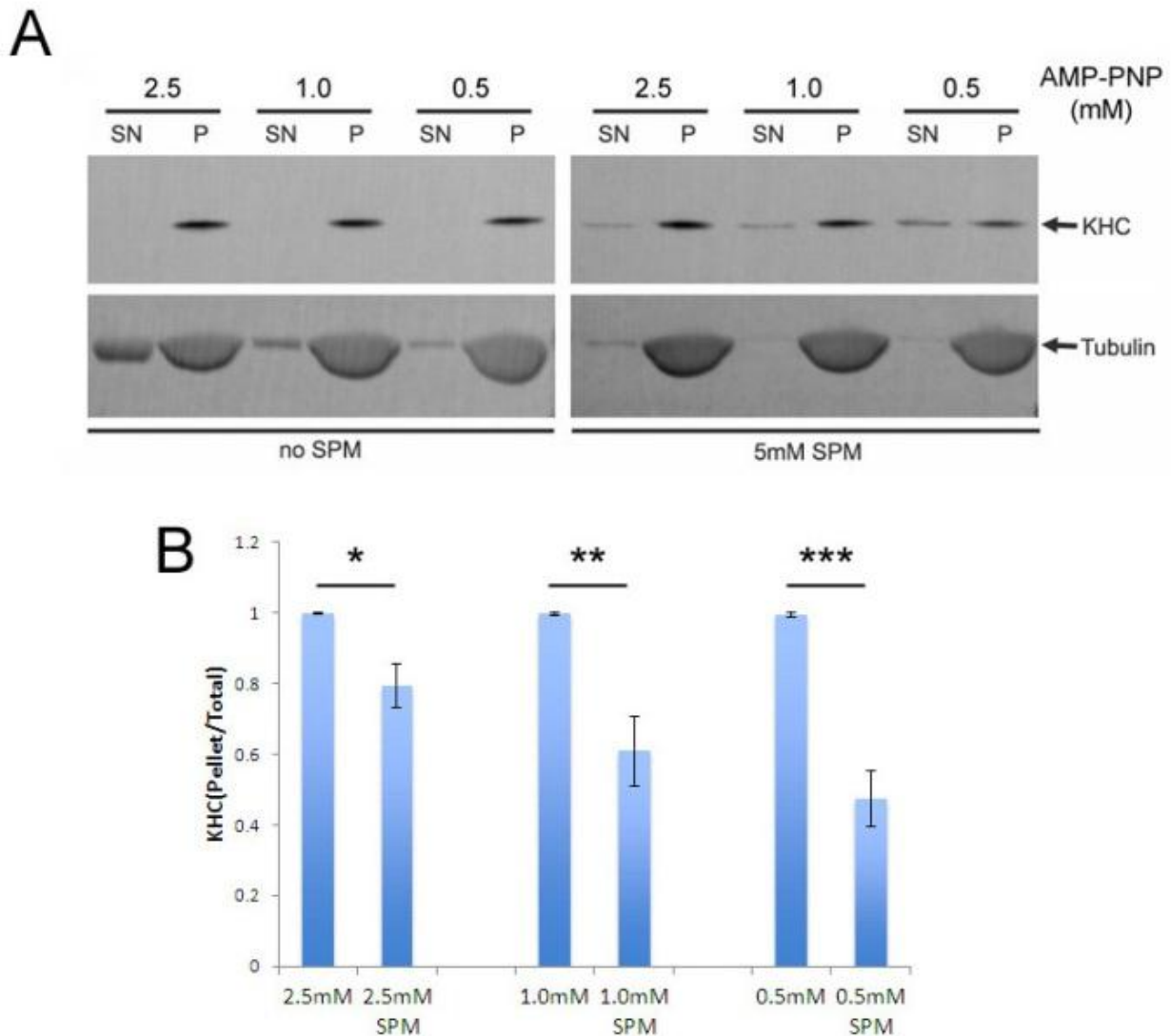


Figure 11. Effect of spermine on kinesin binding to microtubules is negatively correlated with AMP-PNP concentration.

Microtubule pellets (P) and supernatant (SN) fractions were prepared from primary mouse cortical neuron at DIV5 with varying concentration of AMP-PNP, with or without spermine (SPM). Immunoblot analysis using H2 (KHC) and DM1A (Tubulin) antibodies (**A**), and quantification of the immunoblot for KHC (**B**). Enrichment of tubulin in pellet fractions confirms a successful assay. Without SPM, the majority of kinesin came down with the MT enriched pellet for AMP-PNP concentrations from 0.5-2.5 mM. However when SPM was added into the reaction, the amount of kinesin in the pellet fraction decreased as the AMP-PNP concentration decreased. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, $n = 6$, error bar = \pm SEM, student's t -test)

Figure 12

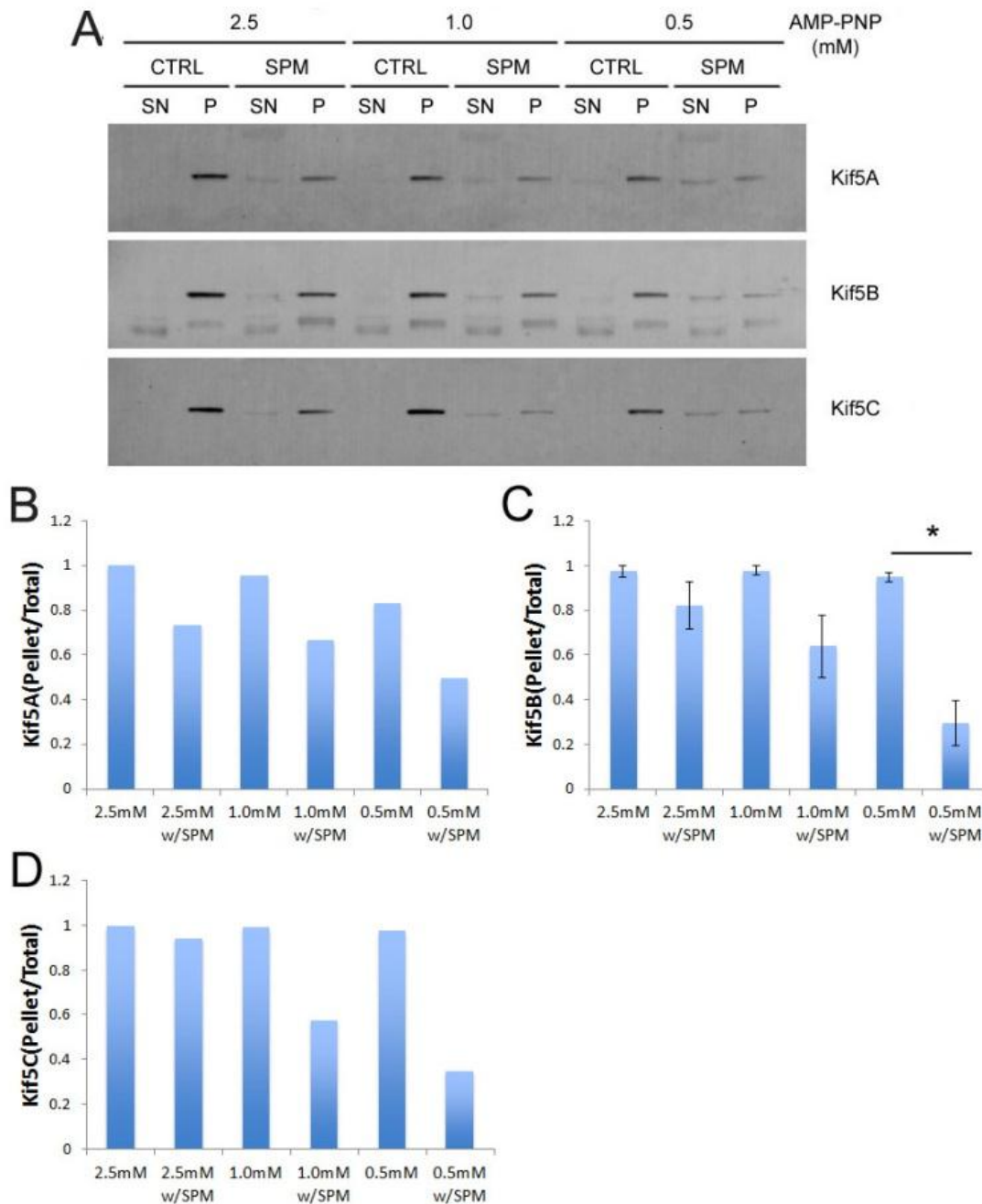


Figure 12 All three isoforms of kinesin exhibit an AMP-PNP concentration dependent effect by spermine.

Samples from figure 11 were analyzed for three isoforms of kinesin-1 by immunoblots (**A**). Quantification of the immunoblot for Kif5A (**B**), Kif5B (**C**), and Kif5C (**D**). All three isoforms of Kinesin-1 were similarly affected. This confirms that AMP-PNP is a potent inhibitor of CK2, and that a high concentration of AMP-PNP masks the effect of SPM to activate CK2. This prevents phosphorylation of Kinesin that leads to its release from MT. (* $p < 0.001$, $n=2$ for Kif5A and Kif5C, $n=4$ for Kif5B, error bar = \pm SEM, student's *t*-test)

samples were analyzed with immunoblot (Figure 12A) and signal intensities were quantified for each isoforms (Figure 12B, C, and D). Same trend was seen with all three KHC isoforms. Lower concentrations of AMP-PNP resulted in more pronounced effects of SPM thus reducing amount of different KHC isoforms in MT enriched pellet.

2.9. Activation of mouse endogenous CK2 by spermine disrupts DHC interaction with DIC.

Perfusion of active CK2 into squid axoplasm inhibits both anterograde and retrograde FAT (Figure 1B and Pigino et al., 2009). The data so far suggest that CK2 impairs anterograde FAT by reducing the binding of the anterograde motor kinesin to the MT tracks. Although CK2 can phosphorylate heavy and intermediate chains of cytoplasmic dynein (Karki et al., 1997), it is not clear if CK2 impairs retrograde FAT by directly affecting cytoplasmic dynein motor complex or as secondary effect to the impairment of anterograde FAT. For example, perfusion of H2, a monoclonal antibody against KHC which does not bind to any of the retrograde motor protein complex, also impairs retrograde FAT (Brady et al., 1990). To determine if activation of CK2 affects binding of cytoplasmic dynein to MTs, MT binding assay samples were analyzed for DHC and DIC with immunoblot (Figure 13A) and signal intensities were quantified (Figure 13B). As controls for tubulin polymerization ATPase activity of dynein, immunoblots for tubulin (Figure 13A lowest panel) and ATP control condition (Figure 13A left two lanes) are shown. Activation of mouse endogenous CK2 by SPM does not affect DHC binding to the MTs, but there is over 40% reduction in the amount of DIC in MT enriched pellet when the lysate was pre-incubated with SPM. Increasing the AMP-PNP concentration did not have an effect on binding of DHC or p150Glued to the MTs regardless of pre-incubation with SPM (Figure 13C top two

Figure 13

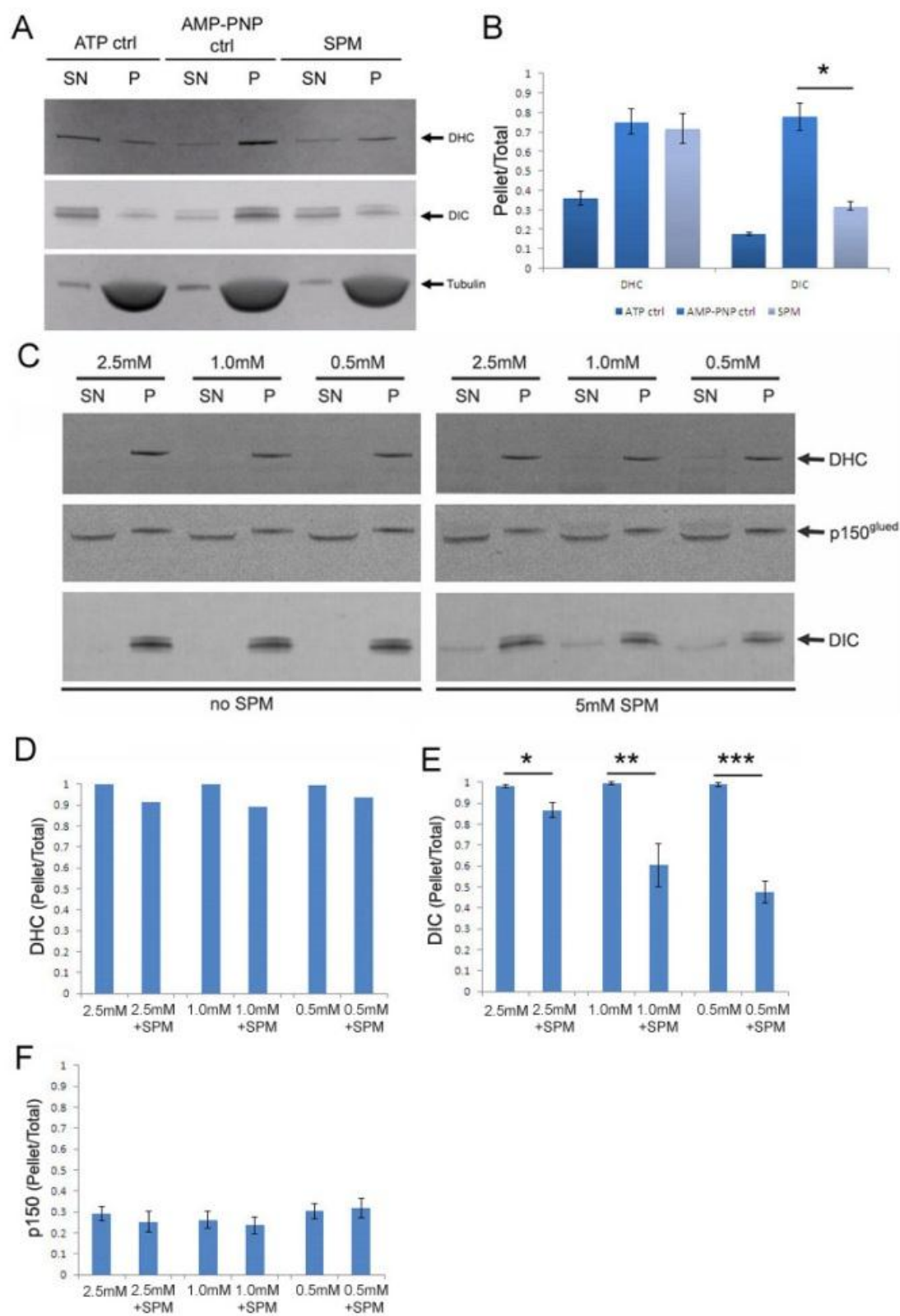


Figure 13. Activation of mouse endogenous CK2 by spermine disrupts DHC interaction with DIC.

Microtubule pellets (P) and supernatant (SN) fractions prepared from primary mouse cortical neuron at DIV5 were analyzed for components of the retrograde motor complex. (A) Immunoblot analysis using Dynein Heavy Chain (DHC), Dynein Intermediate Chain (DIC), and DM1A (Tubulin). Enrichment of tubulin in pellet fraction confirms successful assay. ATP treatment released majority of DHC and DIC into the supernatant, serving as a control for DHC's ATPase activity. The amount of DHC in the MT pellet did not change with the addition of SPM, indicating that activation of mouse endogenous CK2 by spermine does not affect DHC binding to MT. The level of DIC in the MT pellet, on the other hand, was significantly reduced when SPM was added. This implies that activation of CK2 by spermine disrupts the protein-protein interaction between DHC and DIC. (B) Quantification of the immunoblot. (C) Immunoblot analysis of samples from Figure 11C for the components of Dynein complex to examine the effect of SPM on varying concentrations of AMP-PNP. Quantification of DHC and p150^{Glued} from the immunoblot (D and F, respectively) revealed that the concentration of AMP-PNP or the presence of SPM does not affect the MT binding properties of DHC or p150^{Glued}. However, the level of DIC in MT pellet decreased as AMP-PNP concentration decreased. This implicates that a high concentration of AMP-PNP masks the effect of SPM to activate CK2. This prevents phosphorylation of Dynein complex that leads to dissociation of DIC from DHC. The blots also show that level of p150^{Glued} attached to MT is relatively low. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, $n = 5$, error bar = $\pm SEM$, student's t -test)

panels, Figure 13D and F). Although p150^{Glued} has been reported to co-purifies with cytoplasmic dynein, to bind to MTs, and to mediate motility of dynein (Waterman-Storer et al., 1995), it has relatively low affinity to the MTs (~30%) in this assay. The effect of SPM to reduce DIC binding to MTs is negatively correlated with AMP-PNP concentrations while the binding of DHC and p150^{Glued} remain unaffected by SPM or AMP-PNP.

2.10. Differentiated neuroblastoma cell lines have impaired kinesin and dynein binding to microtubules.

We obtained mouse neuroblastoma N2a cell lines stably transfected with wild type human PS1 (hPS1 WT), FAD-linked human PS1 with exon 9 deletion (hPS1 dE9), or empty control vector (vector ctrl) from Dr. Lazarov (Borchelt et al., 1996). The transgenic mice expressing hPS1 dE9 have aberrant kinase activities and show impaired axonal transport in sciatic nerve. We performed MT binding assays with these cell lines that are differentiated to determine if FAD-linked mutation of PS1 results in reduced binding of motor proteins to MTs. The supernatant (SN) and MT enriched pellet (P) were analyzed by immunoblot for KHC, DIC, and tubulin (Figure 14A), and signal intensities were quantified (Figure 14B and C). Surprisingly, there was no difference in the amount of KHC or DIC in the MT enriched pellet between different cell lines. However, CK2 plays an important role in cell cycle regulation, and it is known to be dysregulated in cancer cells (Ahmad et al., 2005). Since neuroblastoma is a cancer of nerve tissue, we compared the results with those obtained using primary mouse cortical neurons (PMCN). The binding of KHC and DIC to the MTs in all three of the N2a cell lines were significantly lower than that of PMCN AMP-PNP control, and significantly higher than

Figure 14

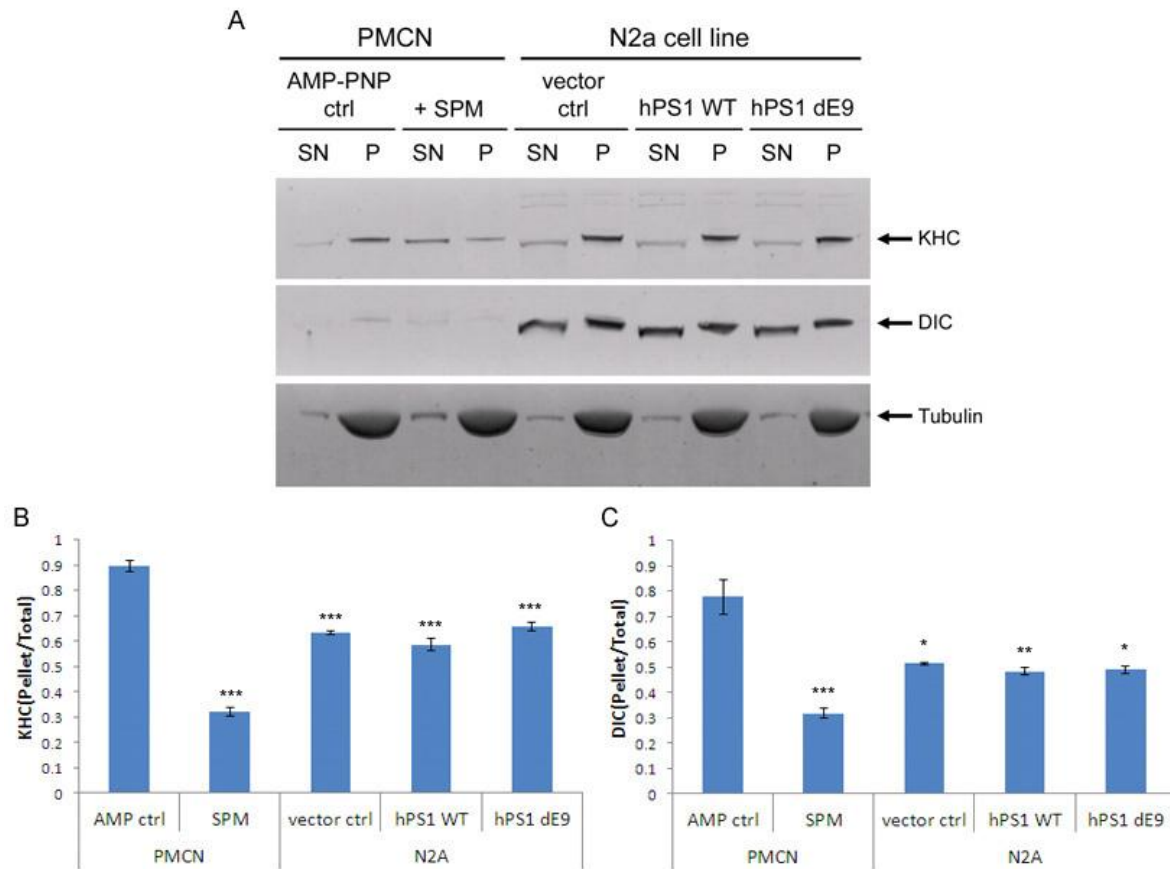


Figure 14. Differentiated neuroblastoma cell lines have impaired kinesin and dynein binding to microtubules.

To examine the effect of inherently high level of CK2 activity in cancer cells and AD related mutation in PS1, microtubule pellets (P) and supernatant (SN) fractions were prepared from differentiated N2a cell lines transfected with empty vector (vector ctrl), wild type human PS1 (hPS1 WT), or FAD-linked PS1 mutation (hPS1-dE9). (A) Immunoblot analysis with primary mouse cortical neurons (PMCN) and N2a cell lines (N2a) using KHC, DIC, and tubulin antibodies. Quantification of KHC and DIC from the immunoblot (B and C, respectively). There was no transfected gene effect on the levels of both KHC and DIC in the MT pellet fraction. When they were compared to the AMP-PNP ctrl, all three cell lines had significantly reduced levels of KHC and DIC in the MT pellet fraction. This suggests that the inherent high level of CK2 activity in neuroblastoma cell lines leads to impaired Kinesin and Dynein binding to MT, and mask the effect of FAD-linked mutation in impaired motor binding. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, $n = 5$ for PMCN, $n = 4$ for N2a, error bar = \pm SEM, student's *t*-test)

PMCN SPM treated lysate. These results suggest that the increased level of basal CK2 activity may mask the effect of FAD-linked mutations on motor protein binding to MTs, and this increased basal activity of CK2 impairs KHC and DIC binding to the MT regardless of the genetic manipulation.

2.11. Activation of mouse endogenous CK2 by spermine does not affect kinesin or dynein binding to synaptic vesicles.

CK2 can directly phosphorylate kinesin light chains (Figure 4A), and phosphorylation of KLC has been shown to result in MBOs to be dissociated from kinesin (Morfini et al., 2002a). We treated lysate from primary mouse cortical neurons with SPM and/or recombinant CK2 to see if activation of CK2 can negatively affect kinesin and dynein binding to the MBOs. Fractions were collected from lysate separated in iodixanol gradient and analyzed by immunoblot for KHC, DIC, and Synaptophysin (Figure 15A). Synaptophysin was used as a marker for the synaptic vesicles, and based on its enrichment in fractions 1 through 4; these were grouped as membrane fractions. The quantification of the immunoblot (Figure 15B) revealed that the amount of KHC and DIC do not change with SPM and /or CK2. The data indicate that increase in CK2 activity does not reduce motor protein binding to synaptophysin positive vesicles.

Figure 15

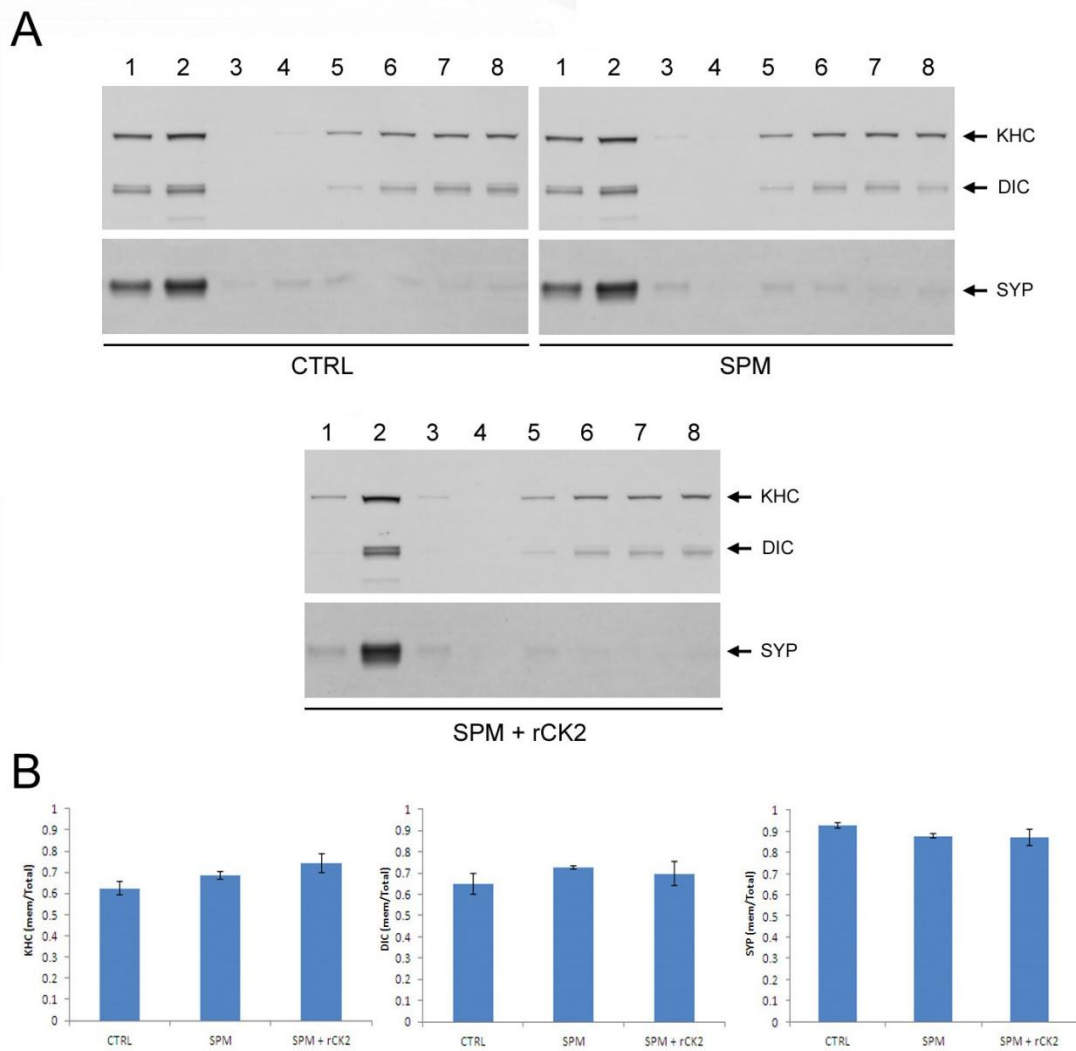


Figure 15. Activation of mouse endogenous CK2 by spermine does not affect kinesin or dynein binding to synaptic vesicles.

Fractions from iodixanol flotation assays were obtained from primary mouse cortical neuron at DIV6. The cell lysate was incubated with itself (CTRL), 5mM spermine (SPM), or 5mM SPM and 500U recombinant CK2 (SPM + rCK2). Fractions were collected from the top to bottom (top fraction = 1, bottom fraction = 8). **(A)** Immunoblot analysis using KHC, DIC, and synaptophysin (SYP). It shows SYP is enriched in fractions 1 to 4 (membrane fraction: mem). **(B)** Quantification of immunoblot showing proportion of KHC, DIC, and SYP in membrane fractions over total protein. Co-incubation of SPM alone or SPM and rCK2 did not affect the levels of KHC and DIC associated with the SYP positive membrane. This suggests that activation of mouse endogenous CK2 by spermine in the cell lysate does not affect Kinesin or Dynein binding to synaptic vesicles. ($n=6$, error bar = \pm SEM, student's *t*-test)

3. To examine impairment of fast axonal transport in an animal model of Alzheimer's disease.

3.1. Presence of FAD-linked PS1 mutation impairs kinesin and dynein binding to microtubules.

Mice with targeted PS1 gene replacement with FAD-linked mutant human PS1 gene (PS1-ki) were used as an animal model of AD in our laboratory. Along with other animal models of AD, these mice exhibit impaired FAT in vivo and in vitro (Kasa et al., 2000; Lazarov et al., 2007; Pigino et al., 2003; Smith et al., 2007)). Lysate from primary cortical neurons of wild type (WT) and PS1-ki mice were processed for MT binding assays. The Immunoblot (Figure 16A) and quantifications (Figure 16B and C) reveal that amount of KHC in the AMP-PNP ctrl MT enriched pellet of PS1-ki mice is significantly reduced compared to WT, and the amount of DIC appeared greatly reduced as well though the statistical analysis could not be done due to lack of sufficient number of replicates. This strongly suggests that kinesin and dynein of PS1-ki mice have impaired binding to the MTs. Of note, the amount of KHC in the ATP control MT pellet fraction from PS1-ki mice is significantly lower than the levels seen with WT although both are below 10% of total protein and within the acceptable range of controls.

3.2. FAD-linked PS1 mutation does not affect kinesin or dynein binding to the synaptophysin positive vesicles.

Next we investigated if FAD-linked PS1 mutation cause impaired FAT by reducing motor protein binding to the MBOs. Lysate from WT, heterozygotic PS1-ki, and PS1-ki mice

Figure 16

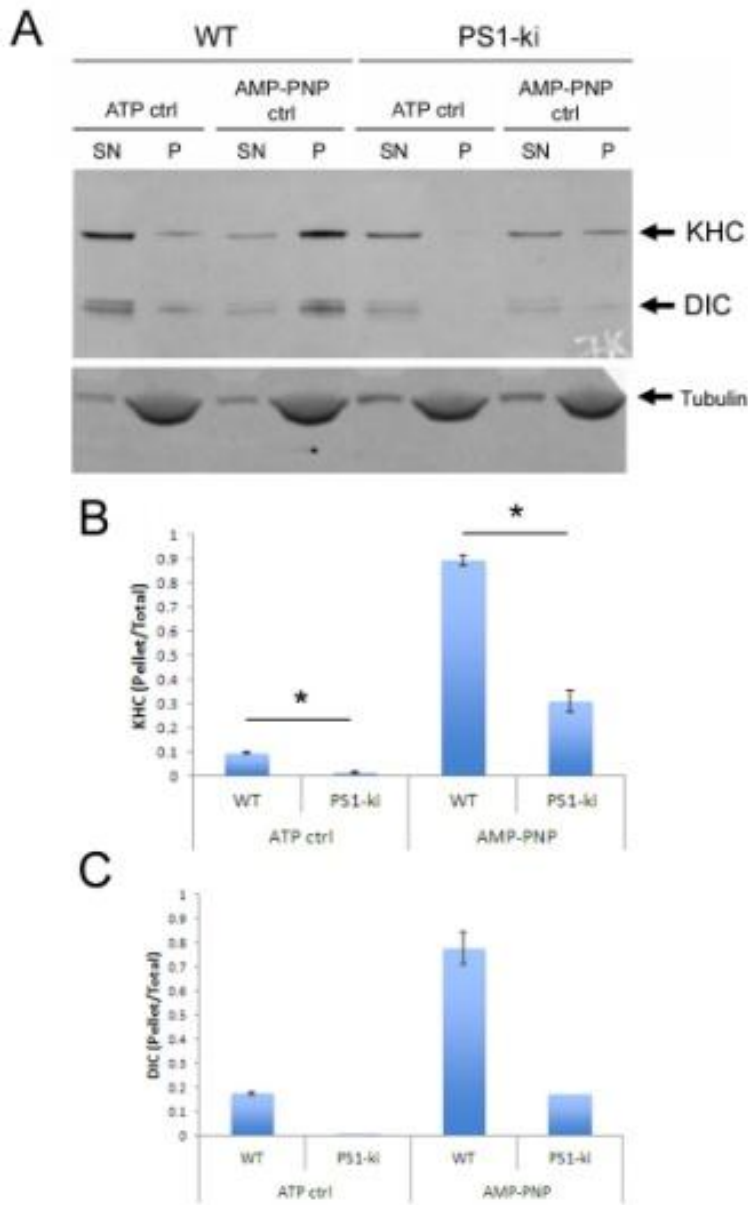


Figure 16. The presence of a FAD-linked PS1 mutation impairs kinesin and dynein binding to microtubules.

Microtubule pellets (P) and supernatant (SN) fractions were prepared from primary mouse cortical neurons (PMCN) of PS1-ki mice at DIV5. **(A)** Immunoblot analysis using KHC, DIC, and Tubulin antibodies. Quantification of the immunoblot for KHC **(B)** and DIC **(C)** compared with WT PMCN. Significantly lower amounts of both KHC and DIC were associated with the MT enriched pellet from PS1-ki mice in both ATP and AMP-PNP conditions. This suggests that neurons of PS1-ki mice have reduced kinesin and dynein binding to the MT, thus contributing to impairment of FAT. (* $p < 0.0001$, $n = 5$ for WT, $n = 4$ for PS1-ki, error bar = \pm SEM, student's *t*-test)

Figure 17

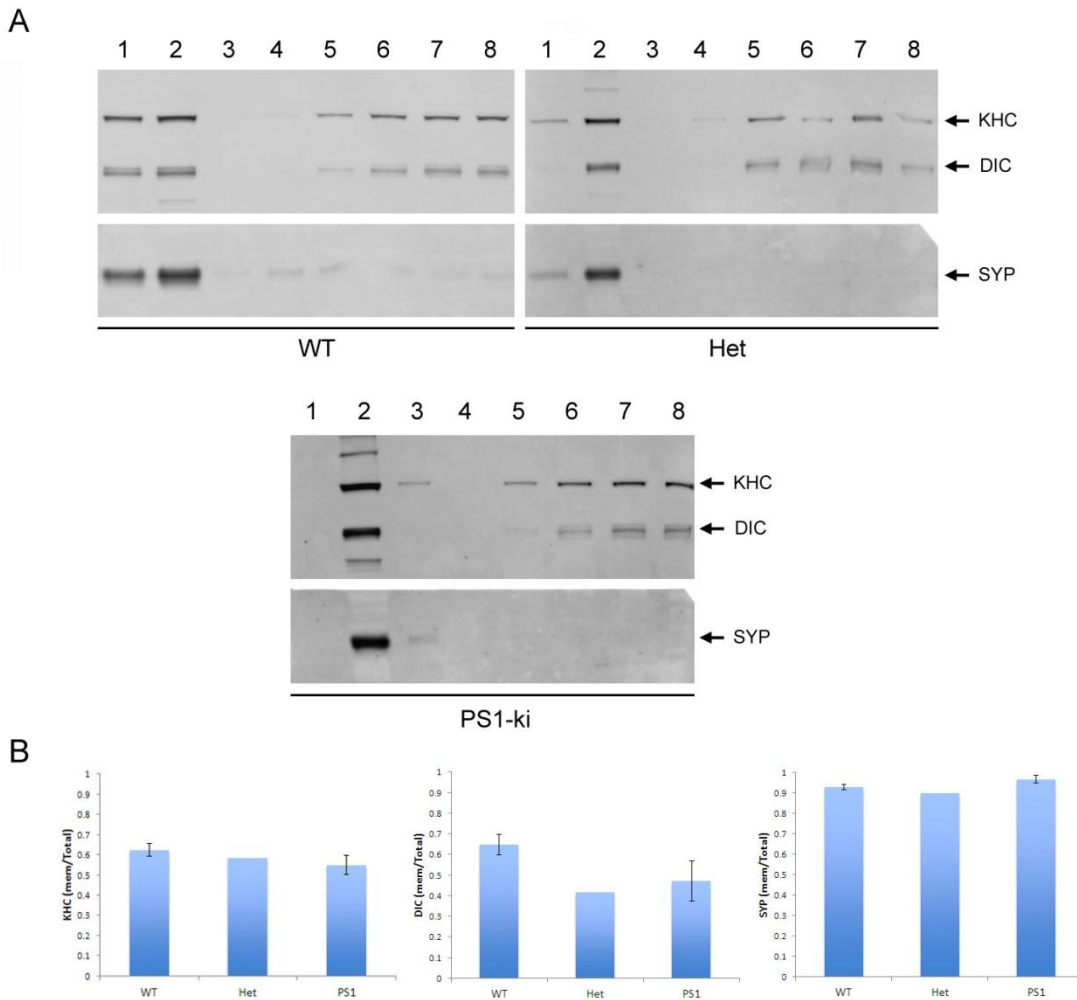


Figure 17. A FAD-linked PS1 mutation does not affect kinesin or dynein binding to synaptic vesicles.

Fractions from iodixanol flotation assays were obtained from primary mouse cortical neuron (PMCN) from WT mice (WT), heterozygotic PS1-ki mice (Het), and PS1-ki mice (PS1) at DIV6. Fractions were collected from top to bottom (top fraction = 1, bottom fraction = 8). **(A)** Immunoblot analysis using KHC, DIC, and synaptophysin (SYP) shows SYP is enriched in fractions 1 to 4 (membrane fraction: mem). **(B)** Quantification of immunoblot showing the proportion of KHC, DIC, and SYP in membrane fractions over total protein. The levels of KHC and DIC in membrane fractions were not significantly different between WT, Het, and PS1. This indicates that kinesin and dynein binding to the synaptic vesicles are not affected by introduction of a FAD-linked PS1 mutation. ($n=6$ for WT, $n=3$ for PS1-ki, error bar = \pm SEM, student's *t*-test)

primary cortical neuron culture were separated on iodixanol gradient, fractions were collected and analyzed by immunoblot for KHC and DIC. As shown in immunoblot (Figure 17A) and the quantification (Figure 17B), over 90% of synaptophysin, a synaptic vesicle marker, is enriched in the membrane fractions, fractions 1 through 4. There is no significant change in the amount of KHC in the membrane fraction between WT, Het, and PS1-ki mice. There is some reduction in the amount of DIC in the membrane fraction of PS1-ki mice compared to the WT ($p=0.11$). These results indicate that FAD-linked PS1 mutant does not significantly impair motor protein binding to the Synaptophysin positive vesicles.

3.3. FAD-linked mutation in PS1 does not alter CK2 nor GSK3 β activities.

Kinase dysregulation in AD is widely reported (Jin and Saitoh, 1995), and CK2 is one of them. In this work, activation of CK2 has been shown to significantly reduce binding of motor proteins to the MTs, but has minimal effect on their binding to the synaptic vesicles. Moreover, our animal model of AD exhibit same profile of impaired motor protein binding to the MTs or the synaptic vesicles. To determine if activation of CK2 is responsible for the altered motor protein binding activities, we evaluated kinase activities of CK2 and GSK3 β , another kinase known to be activated in AD, in PS1-ki mice. GSK3 β usually requires a “priming kinase” to first phosphorylate its substrate, and CK2 is one of the priming kinase (Meijer et al., 2004). Because of a concern that the mechanical homogenization of the cells may activate some kinases, the activities of CK2 and GSK3 β were measured using SLO permeabilized primary mouse cortical neurons to preserve the cytoarchitecture as much as possible. The incorporation of ^{32}P onto appropriate peptide substrates were plotted in figure18. They reveal that the kinase activities of

Figure 18

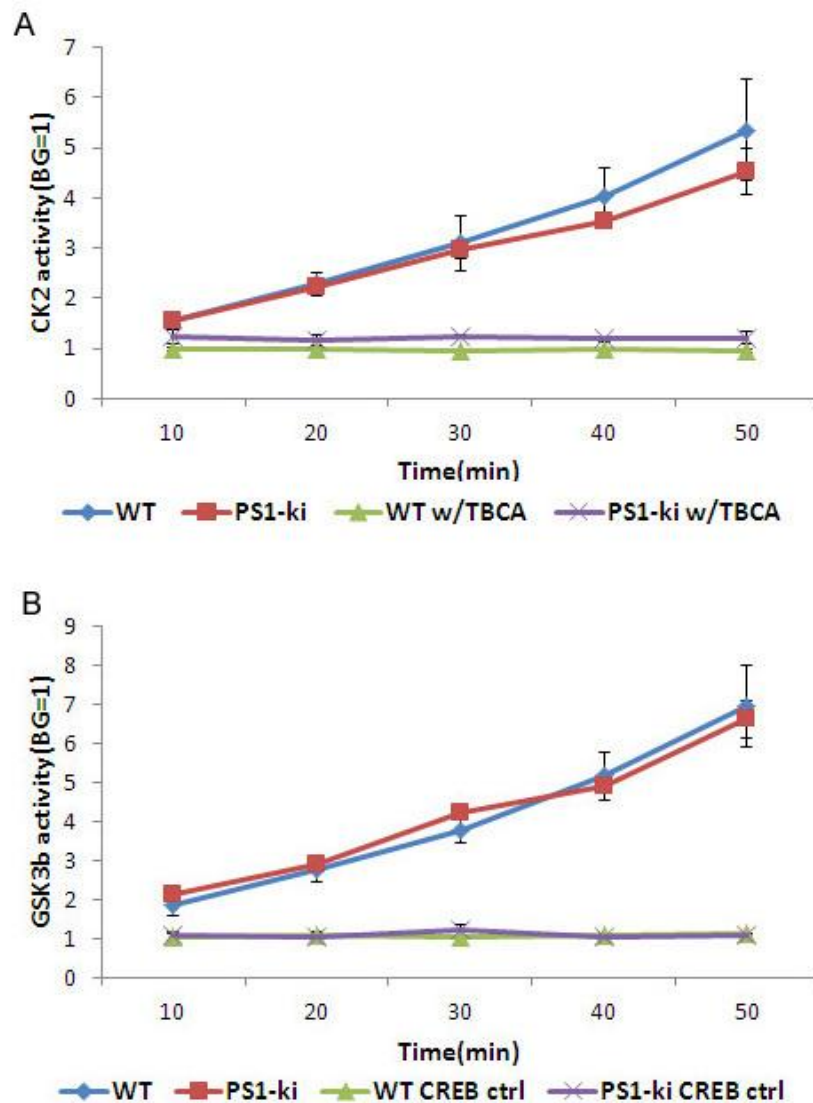


Figure 18. FAD-linked mutation in PS1 does not alter CK2 nor GSK3 β activities.

Kinase activities of CK2 (A) and GSK3 β (B) were measured in Streptolysin-O permeabilized primary mouse cortical neurons at DIV 5. A CK2 inhibitor was co-incubated for CK2 activity assay to show specificity of the assay for CK2. For the GSK3 β activity assay, the CREB control peptide was used instead of pCREB peptide to show specificity. When compared with WT neurons (blue line), neurons from PS1-ki mice (red line) displayed comparable CK2 and GSK3 β activities. ($n=3$, error bar = \pm SEM, student's t -test)

CK2 and GSK3 β (Figure 18A and B, respectively) of PS1-ki neurons were not significantly different compared to the WT neurons.

3.4. There is a region specific activation of CK2 and GSK3 β in 5xFAD mouse brain lysate.

To study if more aggressive animal model of AD displays altered kinase activity, cryopreserved hemi-brain of 4 month old 5xFAD mouse was processed for in vitro kinase assay. 5xFAD mice exhibit accumulation of intraneuronal A β 42 and loss of synaptophysin level by 2 and 4 months, respectively (Oakley et al., 2006). Also these animals show deficits in spatial memory assessed by spontaneous alteration performance in the Y-maze by 4 months of age (Oakley et al., 2006). Tissue lysate from cortex, hippocampus, and cerebellum were prepared and incubated with γ ATP and CK2 peptide substrate to measure the kinase activity. The CK2 activity of 5xFAD is increased in cortex (Figure 19A) and hippocampus (Figure 19B), but not in cerebellum (Figure 19C) when compared to the WT. Also, CK2 activity in cerebellum of both 5xFAD and WT is lower than cortex and hippocampus. The tissue lysate was also analyzed in immunoblot for GSK3 β activity (Figure 19D). Signal intensities of each band were taken and the ratio of inactive GSK3 β (pGSK3 β S9) over the total GSK3 β with KHC as loading control was calculated (Figure 19E). GSK3 β is inactive when Serine 9 is phosphorylated (Sutherland et al., 1993); therefor lower ratio of pGSK3 β S9 means GSK3 β is more active in the lysate. In all three parts examined, the GSK3 β is more active in 5xFAD brain. The difference in pGSK3 β S9 ratio between WT and 5xFAD in cortex and hippocampus is 12~14% and around 6% in cerebellum. These data suggest that CK2 and GSK3 β are activated in a brain region specific manner in 5xFAD mouse brain.

Figure 19

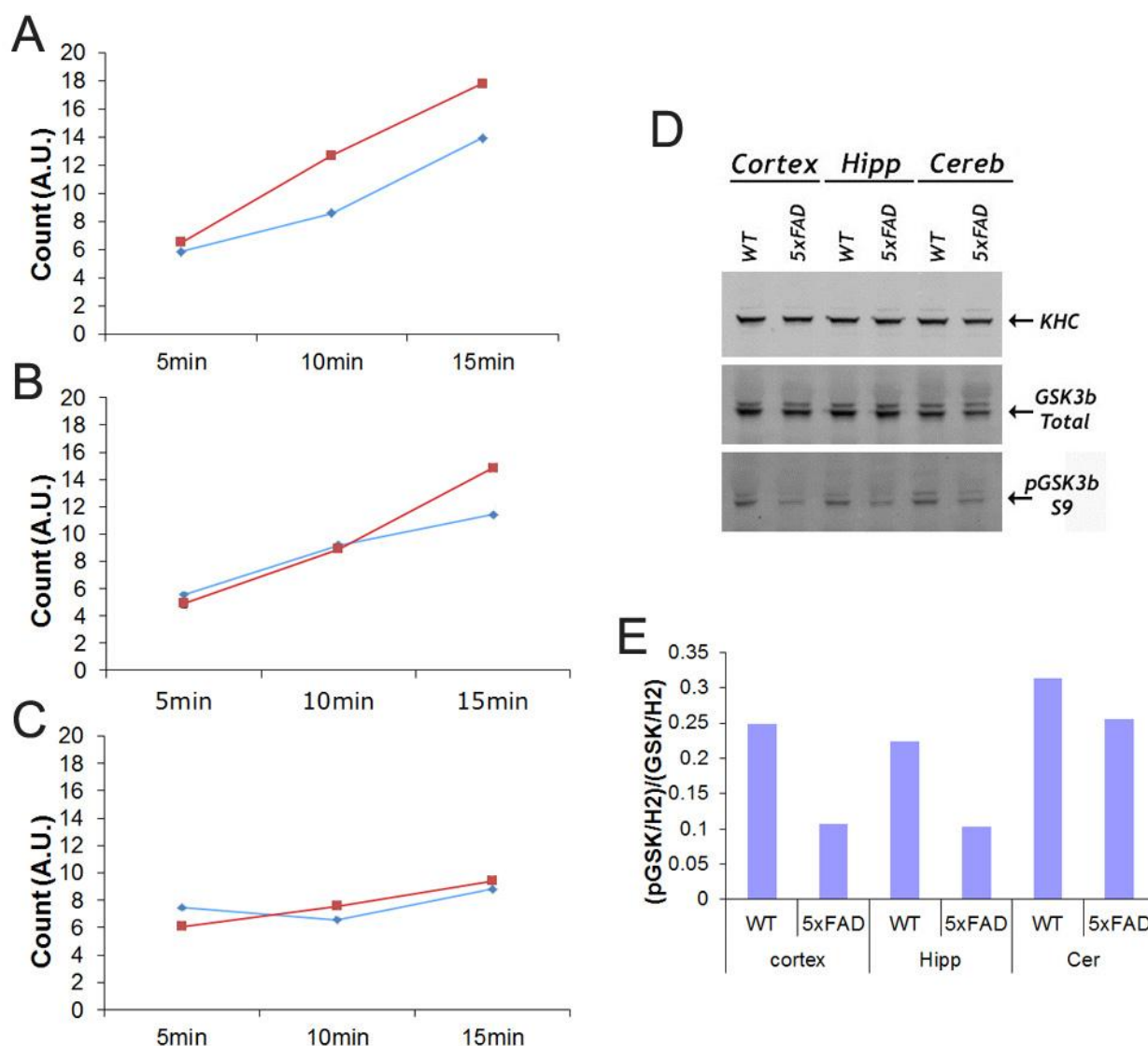


Figure 19. There is a region specific activation of CK2 and GSK3β in 5xFAD brain lysate. Cortex, hippocampus, and cerebellum were dissected from WT C57BL/6 and 5xFAD mice of 4 months. CK2 kinase assays were done with 5 μg of the lysate from cortex (A), hippocampus (B), and cerebellum (C). Aliquots were taken at 5, 10, 15 minutes, and 32 P incorporation onto peptide substrate was counted by liquid scintillation counter. At 15 minutes, 5xFAD lysate showed increased CK2 activities in cortex and hippocampus and not in cerebellum compared to WT lysate. Lysates were also separated in SDS-PAGE gel and probed for KHC, total GSK3β, and pGSK3β_S9 (D). GSK3β that is phosphorylated at serine 9 is considered inactive, therefore the quantification of the immunoblot (E) shows ratio of inactive GSK3β over total GSK3β. In all three parts of the brain examined, GSK3β activities are higher in 5xFAD mouse compared to the WT mouse.

3.5. Activities of CK2 and GSK3 β in cultured primary mouse cortical neurons do not change during the first five days in culture.

Activities of CK2 and GSK3 β are regulated during development (Hur and Zhou, 2010; Sanchez-Ponce et al., 2011; Sanz-Clemente et al., 2010) and aging (Avila, 2011; Ryu et al., 2006). To examine if measurable changes occur in activities of these kinases during the first several days in culture in which cultured neurons go through extensive morphological changes such as neuritogenesis and the neurite extension (Dotti et al., 1988), we performed in vitro kinase assays with SLO permeabilized primary cortical neurons from WT C57BL6 mice at days in vitro (DIV) 1, 2, and 5. CK2 activity is almost identical between DIV 1, 2, and 5 (Figure 20A). GSK3 β activity is slightly higher in cultured neurons at DIV5 than those at DIV1 or DIV2, but the difference is not significant (Figure 20B). These results suggest that CK2 and GSK3 β activities do not change significantly between DIVs 1, 2, and 5.

3.6. Expression of different KHC isoforms in mouse brain during development and aging.

In sporadic forms of AD, aging is the greatest risk factor. In addition to the changes in kinase activities during development and aging, availability of motor protein will be a crucial factor for efficient FAT. Tissue lysates from cortex, hippocampus, and cerebellum of C57BL6 mice at various ages were analyzed in immunoblot for Kif5A, B, and C for relative protein levels to the GAPDH protein levels (Figures 21, 22, and 23, respectively). Immunoblots for Kif5A are shown in Figure 21A as an example. For all KHC isoforms, there are noticeable decreases in protein levels after first few weeks of development. Among the three brain regions examined, the fluctuations in protein levels during development is the smallest in cerebellum. These data imply

that the KHC protein levels are higher early in development, decrease after this period, and stay low for the rest of the adulthood. Also there is brain region dependent regulation of KHC protein level. This supports our hypothesis that expression of motor protein is developmental stage and cell type specific.

Figure 20

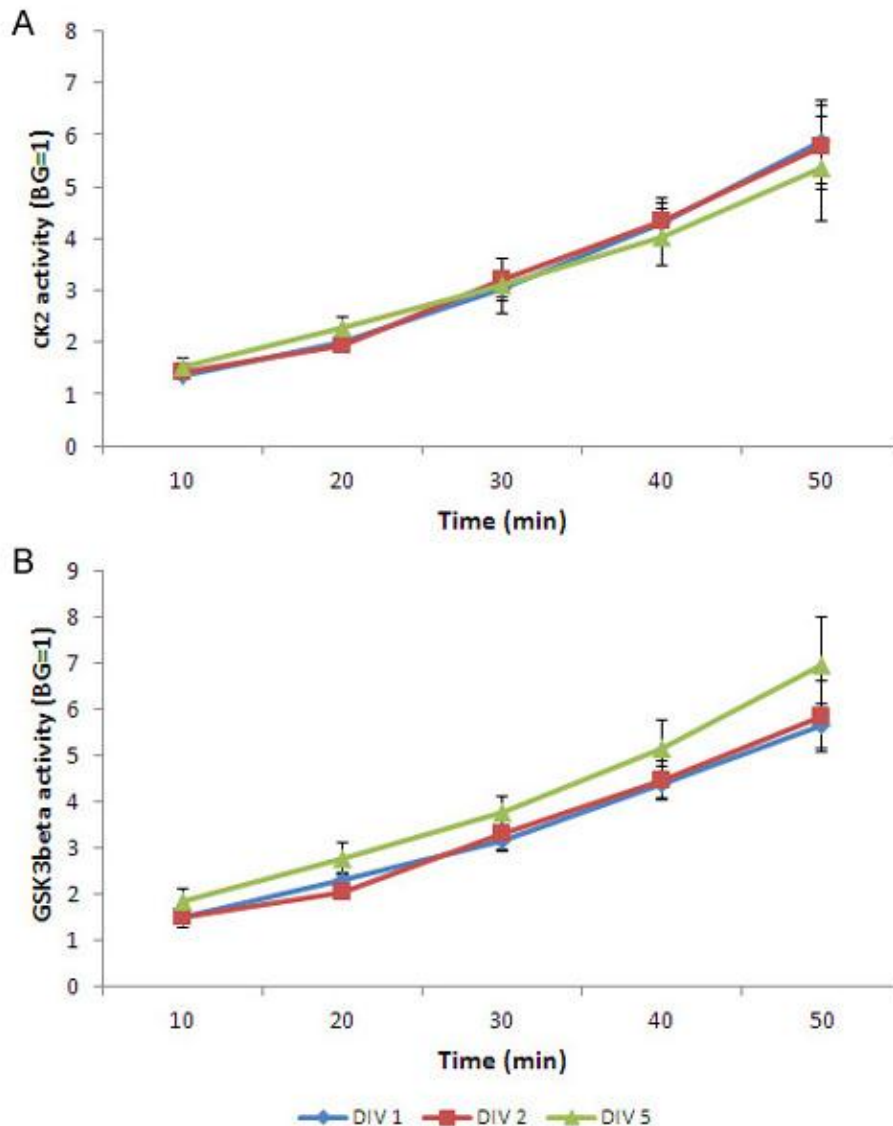
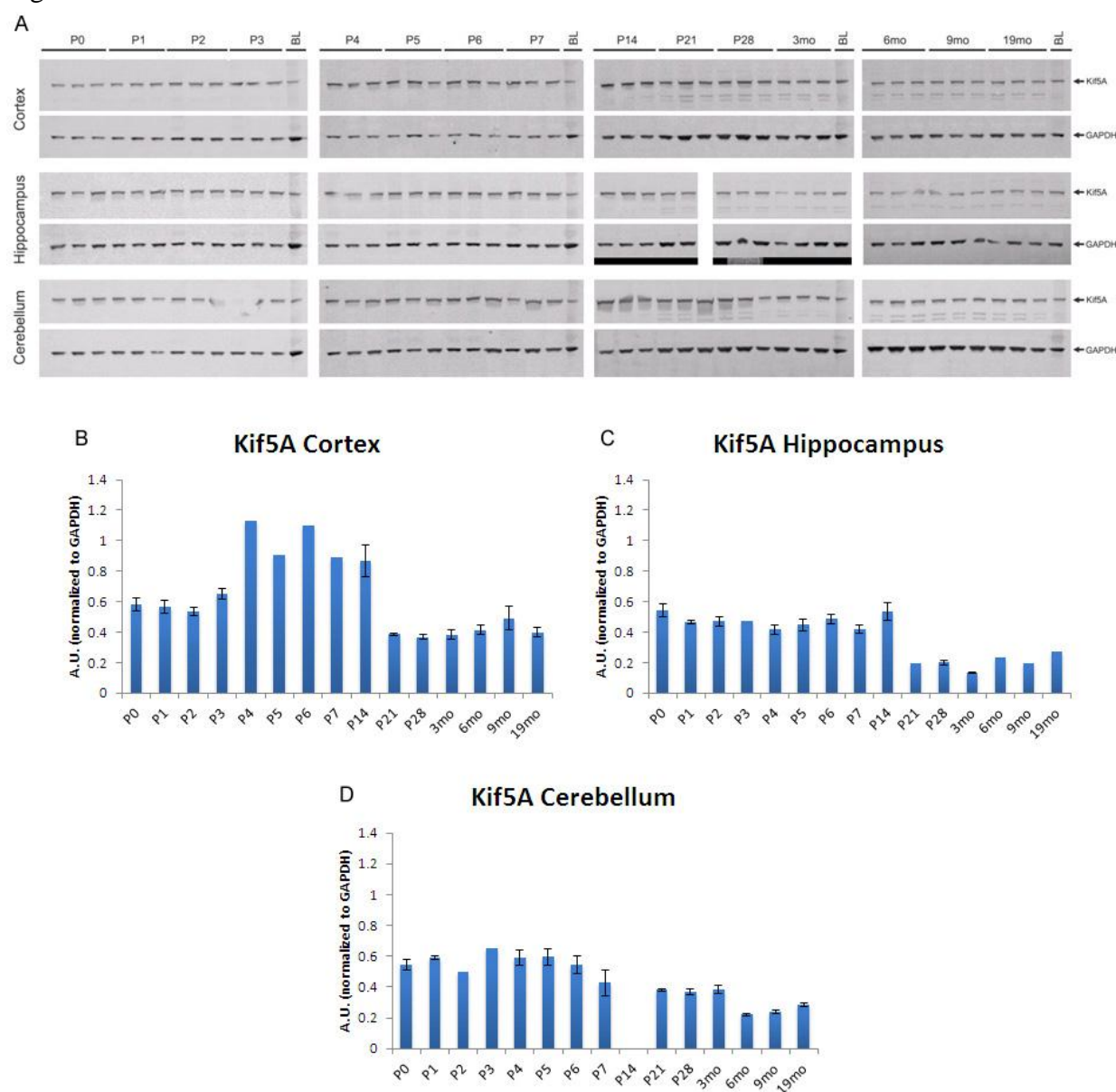


Figure 20. Activities of CK2 and GSK3 β in cultured primary mouse cortical neurons do not change during the first 5 days in culture.

Kinase activities of CK2 (**A**) and GSK3 β (**B**) were measured in Streptolysin-O permeabilized primary mouse cortical neurons at DIV 1, 2, and 5 to examine if kinase activities change during neuritogenesis and the neurite extension period of cultured neurons. A CK2 inhibitor was co-incubated to show specificity of the assay of CK2 activity (data not shown). For the GSK3 β activity assay, CREB control peptide was used instead of pCREB peptide to show specificity (data not shown). The activities of both kinases remain the same from DIV1 to DIV5 despite extensive morphological changes.

Figure 21

**Figure 21. Expression levels of Kif5A in mice during development and aging.**

Tissue lysate were collected from cortex, hippocampus, and cerebellum from WT C57BL6 mice at various post-natal ages. Immunoblot analysis for Kif5A (**A**) was quantified using GAPDH as loading control. For Kif5A, the highest expression is during first few weeks of life in the cortex (**B**), hippocampus (**C**), and cerebellum (**D**). There is noticeable decrease in relative protein level in mice few months or older.

Figure 22

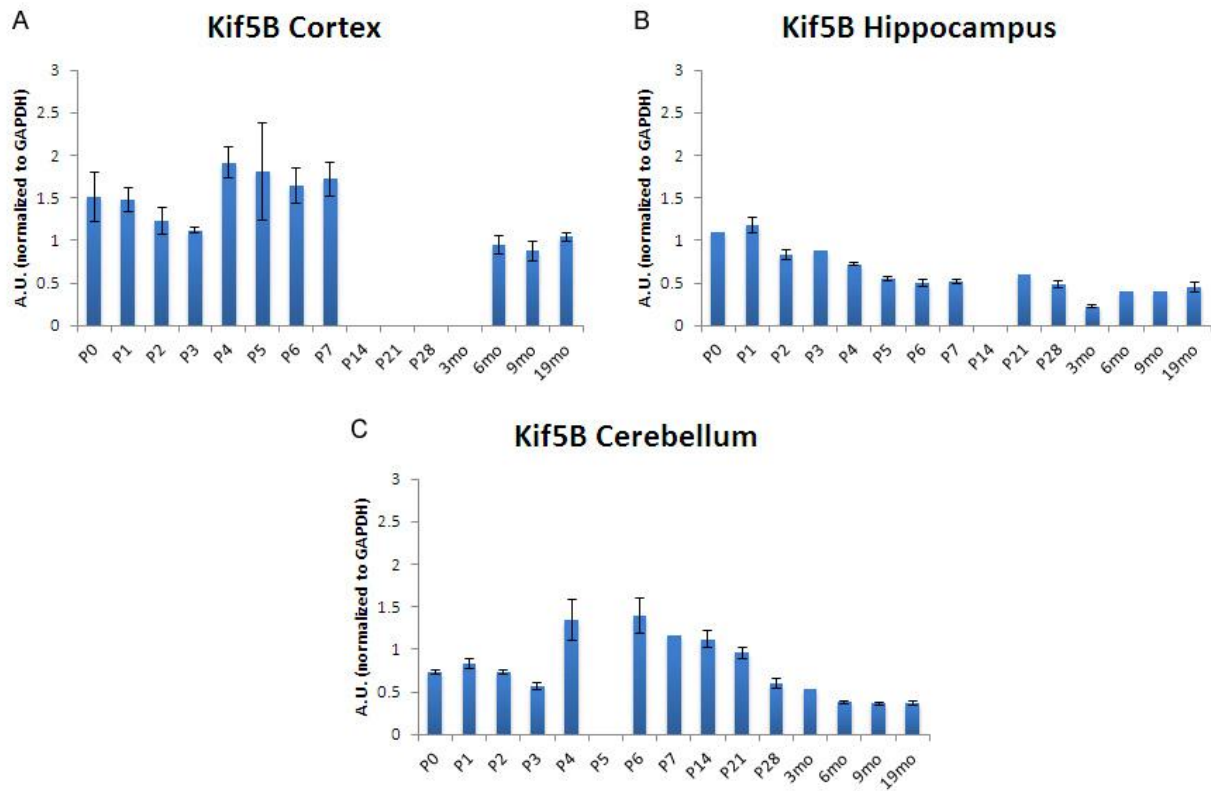


Figure 22. Expression levels of Kif5B in mice during development and aging.

Quantification of Kif5B protein levels relative to GAPDH levels in the cortex (A), hippocampus (B), and cerebellum (C) from immunoblot. Tissue lysate were collected from cortex, hippocampus, and cerebellum from WT C57BL6 mice at various post-natal ages. The relative expression levels of Kif5B are decreased in mice few weeks or older.

Figure 23

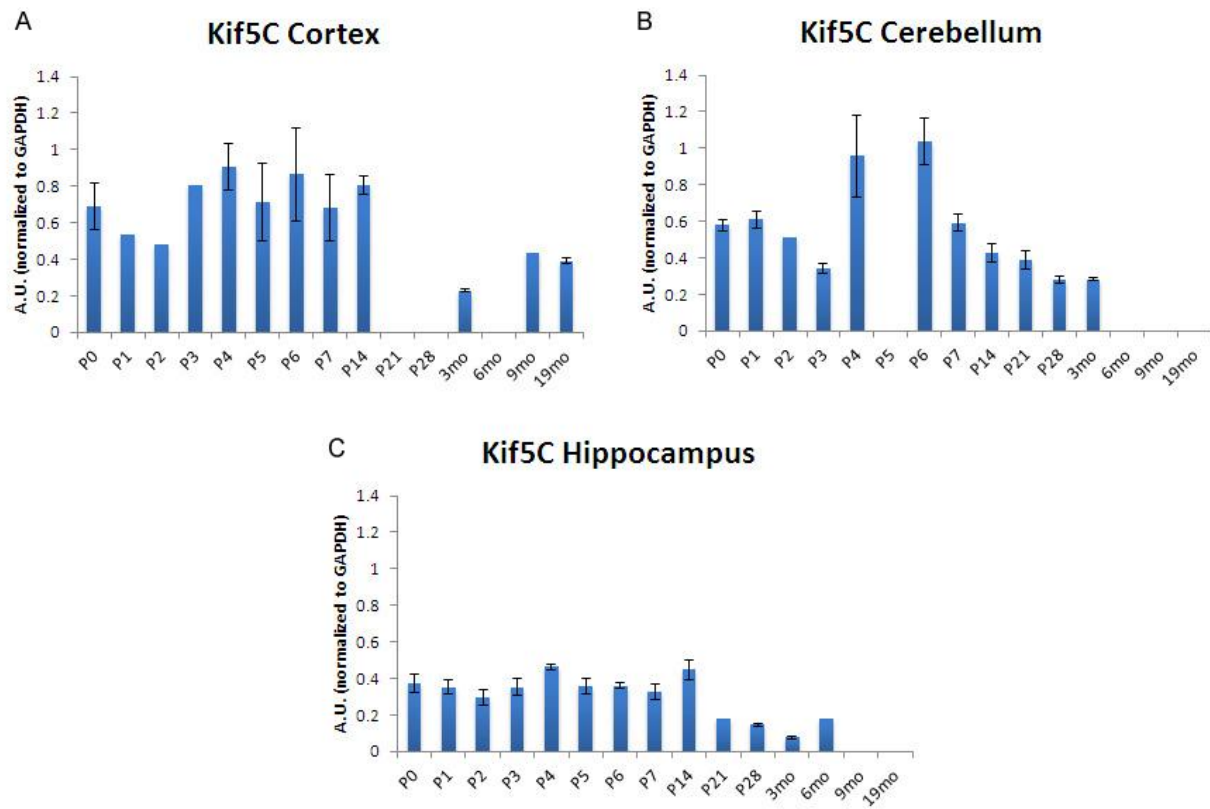


Figure 23. Expression levels of Kif5C in mice during development and aging.

Quantification of Kif5C protein levels relative to GAPDH levels in the cortex (A), hippocampus (B), and cerebellum (C) from immunoblot. Tissue lysate were collected from cortex, hippocampus, and cerebellum from WT C57BL6 mice at various post-natal ages. The relative expression levels of Kif5C decrease in mice as they age.

CHAPTER V

DISCUSSION

1. Apoptosis is a late stage phenomenon in Alzheimer's disease.

In AD the length of time from diagnosis to death can be as long as 20 years during which an individual progressively declines in cognitive and other abilities. This long duration of the disease places tremendous physical, emotional, and financial burden on care givers. It is very important, therefore, to identify the pathological events in early stages of the disease so that therapeutic interventions can be devised and used to stop or even reverse the disease process.

Although neuronal loss in hippocampus and basal forebrain by apoptosis and/or necrosis is one of the pathological features of AD, accumulating data now suggest that neuronal cell death is a late stage phenomenon in AD, and synaptic dysfunction occurs early in AD (Terry et al., 1991). In early days, post-mortem studies were often done with the brains from late stage AD patients in which noticeable neuronal loss is present in the area of the medial temporal lobe (Brun and Englund, 1981). In the past few decades, many studies show that dysfunction and loss of synapses are seen at an early stage of AD and precede apoptotic cell loss. Quantitative analysis of post-mortem brain slices from young patients with AD revealed 25 to 36% loss of synapses in frontal and temporal cortex compared to control subject (Davies et al., 1987). Also there was 25% reduction in immunoreactivity of synaptic markers, synaptophysin, in brain samples with early stages of AD compared with non-demented control subjects (Masliah et al., 2001). Evidences also suggest functional impairment of synapses occurs early in the disease.

Electrophysiological studies on young (1 to 4 months old) transgenic mice over-expressing FAD-linked mutant APP revealed significant impairment of synaptic transmission that preceded plaque formation by several months (Hsia et al., 1999). Another study of post-mortem human brain showed prominent atrophy in white matter in preclinical AD brains with no significant cortical atrophy (de la Monte, 1989). This suggests that axonal and synaptic degeneration occurs early in AD and precedes apoptosis.

Additional data supporting the idea that synaptic failure and axonal degeneration but not neuronal apoptosis is responsible for the clinical progression of neurodegenerative diseases such as AD come from studies in mice deficient in pro-apoptotic protein Bax. In one study, researchers crossed transgenic mouse model of inherited prion disease (Tg(PG14)), a fatal neurodegenerative disease that affects humans and other vertebrates, with Bax knockout mice (Bax $-/-$), and studied their clinical and pathological features. Tg(PG14) mice develop a slowly progressive neurological disorder characterized clinically by ataxia and neuropathologically by deposition of neurotoxic form of prion protein in a synaptic-like pattern, gliosis, and apoptosis of cerebellar granule neurons (Chiesa et al, 2000). Deletion of Bax in Tg(PG14)/Bax $-/-$ mice significantly prevented apoptosis of the cerebellar granule neurons, however it did not rescue synaptic degeneration compared to control Tg(PG14) mice (Chiesa et al., 2005). In addition age of onset of symptoms and duration of the disease were not altered in Tg(PG14)/Bax $-/-$ mice compared to Tg(PG14) mice (Chiesa et al., 2005). Similarly, a mouse model of ALS was crossed with Bax knockout mice to analyze if the death of cranial and spinal motoneurons was an essential component of the pathogenesis of ALS. Although Bax deletion completely prevented loss of motoneurons, it failed to prevent neuromuscular denervation and demyelination of the ventral root motor axons (Gould et al., 2006). Interestingly, Bax deletion delayed the onset of

motor neuron disease slightly, a delay which was correlated with a delay in denervation. This is attributed to functions of Bax distinct from its role in apoptosis (Gould et al., 2006). These studies demonstrate that synaptic dysfunction precedes the cell death, and is responsible for manifestation and progress of neurodegenerative diseases.

Occurrence of synaptic dysfunction followed by axon degeneration progressing from the distal to the proximal axon, and eventual cell death are described as dying-back neuropathies (Coleman, 2005; Morfini et al., 2009a). Alteration of FAT can lead to dying-back neuropathies. As described previously, point mutations in motor proteins that compromise FAT are associated with neurodegenerative diseases with the characteristics of a dying-back neuropathy (Reid et al, 2002, Blair et al, 2006, Puls et al, 2005, Farrer et al, 2009). Dysregulation of FAT is also linked to several late-onset neurodegenerative diseases that exhibit dying-back pattern of neuropathies such as Parkinson's disease (Morfini et al., 2007), poly-glutamine expansion (Morfini et al., 2006; Szebenyi et al., 2003), and AD (Morfini et al., 2002a; Pigino et al., 2003). Molecular mechanism of alteration of FAT in AD, however, is still not fully elucidated.

2. Oligomeric A β 42 is the neurotoxic A β conformation

Since A β was identified as the main component of amyloid plaques in AD, neurotoxicity of A β has been studied extensively. A β exists in three conformationally distinct species; monomers, soluble oligomers, and fibrils. Mounting evidence now indicates that oA β is a major toxic conformation of A β in AD (Deshapande et al, 2006). In addition, a number of studies suggests that accumulation of intracellular oA β plays a role in pathogenesis of AD. For example, intracellular oA β localized in neuronal processes and synaptic compartments was associated with pathological alterations within processes and synaptic compartments in both human AD brain

and animal models of AD (Takahashi et al., 2004a). Also, intraneuronal oA β was correlated with the earliest behavioral deficits in a mouse model of AD (Billings et al., 2005). Furthermore, microinjection of oA β into the cultured primary neurons is reported to be toxic (Zhang et al., 2002). In 2009, we have published data indicating that oA β disrupts one of the critical processes unique to neurons and leads to neuronal specificity, selective vulnerability of specific neuronal population, and late-onset of the disease (Pigino et al., 2009).

3. Oligomeric A β 42 inhibits fast axonal transport through activation of CK2

Here, we present direct evidence that intracellular oA β impairs both anterograde and retrograde FAT. Perfusion of monomeric or fibrillar A β into squid axoplasm at 100nM did not affect FAT (data not shown). Increasing the concentration of fibrillar A β to 2 μ M inhibited FAT, but whether this effect was caused by low levels of oA β contamination (1-2%) or by reduced biological activity for fibrillar A β could not be determined (Pigino et al., 2009). Perfusion of 10 or 100nM of oA β inhibited both directions of FAT to a comparable extent (data for 10nM not shown). Considering the concentration of kinesin in the isolated squid axoplasm is at around 500nM (Brady et al., 1990), it is unlikely that oA β inhibits FAT by directly sequestering kinesin. Rather the effect of oA β is through activation of an enzymatic cascade that regulates FAT. Pathogenic proteins associated with a variety of late-onset neurodegenerative disease such as Kennedy's, Huntington's, and Alzheimer's disease (including androgen receptor, Huntingtin, tau filaments, and PS1) have been found to inhibit FAT through activation of kinases or phosphatases (LaPointe et al., 2009; Morfini et al., 2006; Pigino et al., 2003; Szebenyi et al., 2003). In AD, activities of kinases such as GSK3, JNK, and CK2 are thought to be dysregulated, and these altered kinase activities are highlighted by hyperphosphorylation of neuronal proteins.

By using co-perfusion of kinase inhibitors with oA β , we have determined that CK2 mediates FAT impairment by oA β . GSK3 was excluded from candidate kinase that mediates oA β effect on FAT since it only affects anterograde transport (Morfini et al, 2002) whereas oA β inhibits both directions. Activation of JNK mediates the inhibition of FAT by pathological forms of androgen receptor and Huntingtin (Morfini et al., 2006; Szebenyi et al., 2003), and it has similar profile with the oA β effect on FAT. However, co-perfusion of the SAPK inhibitor SB203580 which inhibits JNK and P38, did not prevent inhibition of FAT by oA β . Activation of CK2 in mediating oA β effects on FAT was confirmed by complete blocking of oA β effects on FAT when isolated squid axoplasm was co-perfused with selective CK2 inhibitors, DMAT and TBCA. Both DMAT and TBCA are highly specific CK2 inhibitors with IC₅₀ of 150nM and 110nM, respectively (Pagano et al., 2007). To examine the possible involvement of secondary kinases in mediating oA β induced FAT impairment, a CK2 specific peptide substrate was co-perfused with oA β . The CK2 specific peptide acts as competitive inhibitor of CK2, binding to the substrate binding site rather than the nucleotide binding site, and it completely prevented oA β effects on FAT. These data confirm that CK2 activation is responsible for inhibition of FAT by oA β . Our *in vitro* experiments and data from other laboratories (Chauhan et al., 1993) suggests that oA β directly increases the activity of CK2 in axons, although the molecular mechanism by which oA β activates CK2 remains to be determined.

4. CK2

CK2 is multifunctional, highly conserved, serine/threonine protein kinase that is ubiquitously expressed in eukaryotic organisms and yeasts (Pinna LA, 1990). It is a tetraheteromeric holoenzyme composed of two catalytic subunits (alpha (α) and alpha prime

(α')) and two regulatory beta (β) subunits. The holoenzyme may have one of three configurations $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, and $\alpha'_2\beta_2$ although the functional significance of these differences are not well understood. It is found in many cell compartments such as cytoplasm (Kandror et al., 1989), nucleus (Krek et al., 1992), and plasma membrane (Pyerin et al., 1987). CK2 is essential for cell survival as knocking out CK2 α and CK2 α' or CK2 β subunit gene in mice leads to early embryonic lethality (Buchou et al., 2003; Padmanabha et al., 1990). CK2 is involved in cell cycle progression (Wang et al., 2003), protein transcription (Guo et al., 1999a), and circadian clock (Sugano et al., 1998). There are over 300 substrates identified to be phosphorylated by CK2 (Meggio and Pinna, 2003), but physiological mechanisms of regulation remain elusive. For a majority of protein substrates, CK2 β subunit enhances phosphorylation activity of CK2 α subunit; however, it suppresses phosphorylation of a few substrates such as calmodulin, and murine double minute clone 2 (MDM2) protein (Meggio et al., 1994). Moreover, interaction of CK2 β has shown to increase heat stability, proteolysis resistance, and optimal ionic strength of CK2 α (Antonelli et al., 1996; Guerra and Issinger, 1999). Polyamines including putrescine, spermidine, and spermine, are known to be potent activators of CK2 (Hathaway and Traugh, 1984b). Its activity has been found to be regulated by cell cycle stages (Wang et al., 2003) developmental stages (Diaz-Nido et al., 1994), and synaptic activity (Charriaut-Marlangue et al., 1991). Dysregulation of CK2 has been linked to wide array of diseases such as cancer (Guerra et al., 1999), cardiovascular disease (Hauck et al, 2008), infectious diseases (Chen et al, 2008, Marjuki et al, 2008), and AD (Iimoto et al., 1990). Increase in immunoreactivity of CK2 has been shown to precede NFT formation in human AD brain (Masliah et al., 1992). How aberrant CK2 activity plays a role in pathogenesis of AD was elusive. Here we provide evidences that CK2 activation leads to inhibition of both directions of FAT by reducing binding of motor proteins to MT.

5. Molecular mechanism of fast axonal transport impairment by CK2

Kinases may alter FAT by two different mechanisms; dissociation of motor proteins from MT or from cargoes. Regulation of kinesin-based FAT has been well studied. For example, JNK phosphorylate KHC at Serine176 (Morfini et al., 2009b) and reduces binding of kinesin-1 to MTs (Morfini et al., 2006). Alternatively, phosphorylation of KLCs by CK2 and GSK3 β inhibits the ability of kinesin-1 to bind cargoes (Morfini et al., 2002b).

Previous data (Donelan et al., 2002) showed, and our current data confirmed that CK2 can phosphorylate both KHC and KLC. However, because of the complex nature of its activity, it is necessary to examine whether CK2 inhibits FAT by directly phosphorylating kinesin-1 or if CK2 phosphorylates downstream kinase that affects FAT. A mouse monoclonal antibody 63-90 was developed in our laboratory using first 50 amino acids of KLC1 as immunogen (Stenoien and Brady, 1997), and it preferentially recognizes dephosphorylated KLCs (Pigino et al., 2003). Incubation of kinesin-1 enriched fraction isolated from mouse with ATP reduced 63-90 immunoreactivity of KLCs with 63-90, and conversely pretreatment of the same fraction with alkaline phosphatase increased 63-90 immunoreactivity (Pigino et al., 2003). These 50 amino acids contain putative phosphorylation sites by GSK3 and CK2. We have determined that 63-90 preferentially recognizes KLCs that are dephosphorylated at the CK2 site which is a priming site for GSK3 phosphorylation. This enabled us to evaluate the CK2-linked phosphorylation state of KLCs. The squid axoplasm perfused with oA β revealed reduced 63-90 immunoreactivity indicating that activation of CK2 by oA β directly modifies kinesin and inhibits FAT. Reduced 63-90 immunoreactivity has previously been reported in different animal models of (Lazarov et al., 2007; Pigino et al., 2003). This suggests that activation of CK2 is a common occurrence in the pathogenesis of AD.

CK2 phosphorylates KHC at serine 175. Recombinant proteins that contain the first 584 amino acids of KHC were generated. Using site-directed mutagenesis, a non-phosphorylatable KHC584 S175A and pseudo-phosphorylated KHC584 S175D were generated. MT binding assay with S175A and S175D revealed that the mutation which mimics phosphorylation of KHC at S175 inhibits KHC binding to MTs. A comparable effect of reduced kinesin binding to MT was observed by using CK2 activator, SPM, with lysate from primary mouse cortical neurons. These data collectively suggest that phosphorylation of KHC by CK2 reduces kinesin binding to MT, and inhibit kinesin-1 based anterograde transport.

Three isoforms of KHC have been identified in mouse. They form homodimers, and in combination with KLCs they are associated with biochemically distinct populations of MBOs (DeBoer et al., 2008). Interestingly, Kif5B lacks serine at 175. However, immunoblot analysis of different KHC isoforms from aforementioned MT binding samples revealed that all KHC isoforms are equally affected by SPM-induced CK2 activation. This raises the possibility that a serine or threonine other than S175 is modified by CK2 and negatively affects KHC binding to MT. The most likely alternate S176 may be phosphorylated by CK2 in the absence of S175, and mimics KHC phosphorylation by JNK. Mass spectrometry analysis of full length Kif5B phosphorylated by CK2 will provide answers to these possibilities. Also, fairly high concentration of SPM (5mM) may affect other cellular enzyme to reduce KHC binding. Lower SPM concentration may be tested to address this concern.

AMP-PNP, a non-hydrolyzable analogue of ATP binds to the nucleotide binding pocket of KHC and stabilizes kinesin-1 interaction with MT. However, it also binds to the nucleotide binding pocket of various kinases present in cell lysate including CK2 and inhibits their activity. When MT binding assay was performed with 2.5mM of AMP-PNP, the SPM-induced reduction

of kinesin binding to MT was smaller compared to assays with 1.0mM or 0.5mM of AMP-PNP. Since AMP-PNP is a potent kinase inhibitor the cell lysate was sequentially incubated first with SPM followed by with AMP-PNP to minimize the inhibitory effect of AMP-PNP on CK2.

Regulation of cDyn processivity by kinase activity is still not fully elucidated. Increased phosphorylation of DHC and DIC in rat hepatocytes by phosphatase inhibitors resulted in inhibition of cDyn ATPase activity (Runnegar et al., 1999). In contrast, perfusion of PKC catalytic subunit increased retrograde FAT in squid axoplasm without affecting anterograde transport (Morfini et al., 2007). Both decrease and increase of cDyn-mediated retrograde FAT can be deleterious to survival of neurons. If retrograde FAT is impaired, waste materials cannot be degraded properly and signaling endosomes do not reach cell body to elicit appropriate response. On the other hand, excessive retrograde FAT with normal anterograde FAT results in untimely return of transported cargoes, and synaptic terminal is depleted of materials needed for its function. It is known that phosphorylation of one or more cDyn components alter its function, but molecular mechanisms of how phosphorylation leads to differential effect on cDyn processivity is uncertain. Kinases that phosphorylate cDyn include PKC, polo-like kinase, PKA, and CK2.

We analyzed the MT binding samples for different components of cDyn although the assay is optimized for kinesin binding to MT. The results suggested a novel mechanism for inhibition of cDyn-based retrograde FAT by activation of CK2. When primary mouse cortical neuron lysate was incubated with CK2 activator, SPM, level of DHC in MT enriched pellet fraction did not change, but the level of DIC was significantly reduced. This suggests activation of CK2 disrupts DHC interaction with DIC and leads to impairment of retrograde FAT. AMP-PNP concentration dependent effects of SPM were also observed for DIC. This supports the idea

that disruption of DHC-DIC interaction is mediated by kinase activities. It should be noted, that perfusion of monoclonal antibody against KHC also inhibits cDyn-based retrograde FAT (Brady et al, 1990). Although the mechanism is not clear, it is possible that inhibition of cDyn-based retrograde FAT by CK2 is secondary to inhibition of kinesin-based anterograde transport. *In vitro* assays using purified kinesin-free cDyn may be suited to address this question.

MT binding assays using a mouse neuroblastoma cell line transfected with an FAD-linked PS1 (PS1 Δ E9) mutation yielded a very interesting result. Although immunoblot analysis of mice harboring this mutation suggests higher level of kinesin phosphorylation by CK2 (Morfini et al., 2007), the level of kinesin in the MT-enriched pellet was not different from WT or empty vector transfected controls in this experiment. However, CK2 activity may already be elevated in cancer cells, so it is possible that the effects of a PS1 mutation on CK2 activity is minimal in this tumor derived cell line. In support of this interpretation, levels of both KHC and DIC in MT-enriched pellet are significantly lower when compared to primary neurons. Kinase assays using these cells is essential to test this possibility.

Incubation of lysates from cultured primary mouse cortical neurons with SPM with or without recombinant CK2 did not alter kinesin and cDyn binding to synaptophysin positive vesicles. This is in contrast to previously published data that CK2 reduces kinesin binding to MBOs (Pigino et al., 2009). This could be due to differential effects of CK2 between the species as the former study was done with isolated squid axoplasm. Further, the previous study used TrkB as a vesicle marker. TrkB and synaptophysin are transported in different population of cargo vesicles (DeBoer et al., 2008), thus reduction of kinesin associated with MBOs by CK2 may be cargo-type specific. In addition, incubation of SPM to activate CK2 suppresses GSK3 β (figure 7C), a kinase that has been shown to promote dissociation of kinesin from cargo vesicles

(Morfini et al., 2002b). It would be necessary to evaluate the effect of CK2 activation on kinesin-vesicle binding independent of other phosphotransferase activity.

6. Impairment of fast axonal transport in an animal model of Alzheimer's disease

Impairment of FAT in animal models of AD has been widely reported by our and other laboratories. One study administered manganese solution intranasally into Tg2576 mice overexpressing FAD-linked human APP mutant and tracked the time to reach olfactory bulb with MRI (Smith et al., 2007). The authors found that the transport rate of manganese in Tg2576 mice decreased compared to WT mice as A β levels increased without plaque formation (Smith et al., 2007). This strengthens the concept that impairment of FAT begins in the initial stages of the disease and precedes formation of amyloid plaque.

We used PS1-ki mice to examine alterations in kinase activities and motor protein functions. This mouse was generated by replacing exon 5 of the mouse wild-type PS1 gene with a mutant human form containing the FAD-linked M146V mutation (Guo et al., 1999b). These mice develop deficit in spatial memory by 3 months of age (Sun et al., 2005). Immunoblot analysis of brain homogenates, fibroblasts, and cortical cultures show decreased immunoreactivities for 63-90 (Pigino et al., 2003) indicating CK2 activity is altered in these mice. Initially we performed MT binding assays with brain lysate from aged PS1-ki mice. This resulted in no difference in the level of KHC or DIC in the MT-enriched pellet with the age-matched WT mice (data not shown). The brain lysate contains proteins from neurons, astrocytes, oligodendrocytes, and many other cell types, and neuron specific effect of mutant PS1 on kinesin binding to MT may be diluted. In order to obtain pure neuronal lysate, we have prepared primary

cortical neuron culture and used them for MT binding, iodixanol density gradient vesicle flotation, and in vitro kinase assays.

Results from MT binding and iodixanol flotation assays with PS1-ki mice concur with data from SPM treated WT neurons. Both KHC and DIC binding to MT were significantly decreased in PS1-ki mice. This strongly suggests that impairment of FAT in PS1-ki mice is mediated through activation of endogenous CK2. It is noteworthy that a previous study with this animal, binding of kinesin to MBO was shown to be decreased in fibroblasts (Pigino et al., 2003), but our data did not show any reduction on kinesin or cDyn associated with MBOs containing synaptophysin. This could be due to a cell type specific activity of CK2 or isoform differences in the kinesin on these vesicles. For example, in insulin-secreting β cells only the KHC is phosphorylated by CK2 (Donelan et al., 2002), but in neurons both KHC and KLC are phosphorylated by CK2. It is still puzzling that kinesin binding to MBO was not affected by mutant PS1 since phosphorylation of KLCs by GSK3 results in release of kinesin from MBOs, and over-activation of GSK3 is a widely accepted phenomenon in AD pathology. It should also be noted that AD takes decades to manifest in humans, and using neurons cultured from embryonic brain may not fully recapitulate dysregulation of kinases.

In vitro kinase assays with SLO permeabilized primary cortical neurons from WT and PS1-ki mice at DIV6 revealed no differences in CK2 and GSK3 activities between WT and PS1-ki mice. In high density cultures, neurons reach stage 5 (Dotti et al., 1988) during DIV5~7. At this stage, neurons project extensive dendrites and a single long axon, and start to form synapses with neighboring cells. Due to the complexity and extensiveness of list of substrates for CK2, activity of CK2 is tightly regulated through its intracellular localization and interacting proteins. Data supporting the role of cellular compartment and cell type specific activation of CK2 for

specific functions comes from the study that downregulation of chromatin- or nuclear matrix-associated CK2 by 30-40% was sufficient to induce potent apoptosis in cancer cells even when a smaller reduction was noted in CK2 activity in the total lysate. (Ruzzene et al., 2002; Slaton et al., 2004). Moreover, there is no correlation between incidence of cancer and AD even though both diseases may be characterized by aberrant CK2 activity. It is crucial to identify the specific pool of CK2 that are dysregulated.

In AD, neurons in hippocampus and basal forebrain are more severely affected and neurons in the cerebellum remain relatively spared. To examine brain region specific activities of CK2 and GSK3 in a more aggressive animal model of AD, we obtained brain hemispheres of 4 months-old 5xFAD mouse from Dr. LaDu. 5xFAD mice possess 3 FAD-linked mutations of APP and 2 FAD-linked mutations of PS1, and accumulation of intraneuronal A β begins at 1.5 months of age (Oakley et al., 2006). 5xFAD mice exhibit progressive loss of synaptic markers with age, and upregulation of kinases such as CDK5 and p25 as early as 3 months of age (Oakley et al., 2006). In vitro kinase activity assay using the tissue lysate from this animal model of rapid progression of amyloid pathology revealed region specific activation of CK2 and GSK3. Both CK2 and GSK3 activities were elevated in cortex and hippocampus but not in cerebellum. This region specific activation of kinases matches with region specific pathology of AD. 4 months-old 5xFAD mouse can be considered to have amyloid pathology comparable with moderate to severe AD since deposition of amyloid plaques begins at 2 months of age (Oakley et al., 2006), but lacks tau pathology. This suggests that dysregulation of kinase activities plays a role in not only the pathogenesis but also progression of AD. Repeating the kinase assay with younger 5xFAD mice possibly 1~2 months of age would help identify early changes of kinase activities.

7. Changes in kinase activities and availabilities of motor proteins contribute to late-onset nature of AD

In FAD, an individual is born with a FAD-linked mutation, but he/she does not suffer from any significant developmental anomalies, except for individuals with Down syndrome. He/she will have a normal life for decades, but begins to suffer from progressive decline of cognitive abilities starting in mid-life. An explanation for this apparent paradox can be found in changes in FAT capabilities in aging nervous system. During aging, the capability of FAT, defined as the amount of materials transported and velocity of the transport both decline (Fig. 24 green line). The rate of decline in FAT capabilities is slow and may not produce a phenotype for years, until levels fall below what is needed to maintain synaptic function. Thus, it may take years for neurodegenerative phenotypes to appear. Once the capability of FAT goes below the threshold to support neuronal function and survival (Fig. 24 shaded area), neurons degenerate via dying-back neuropathy. People are born with FAT

Figure 24

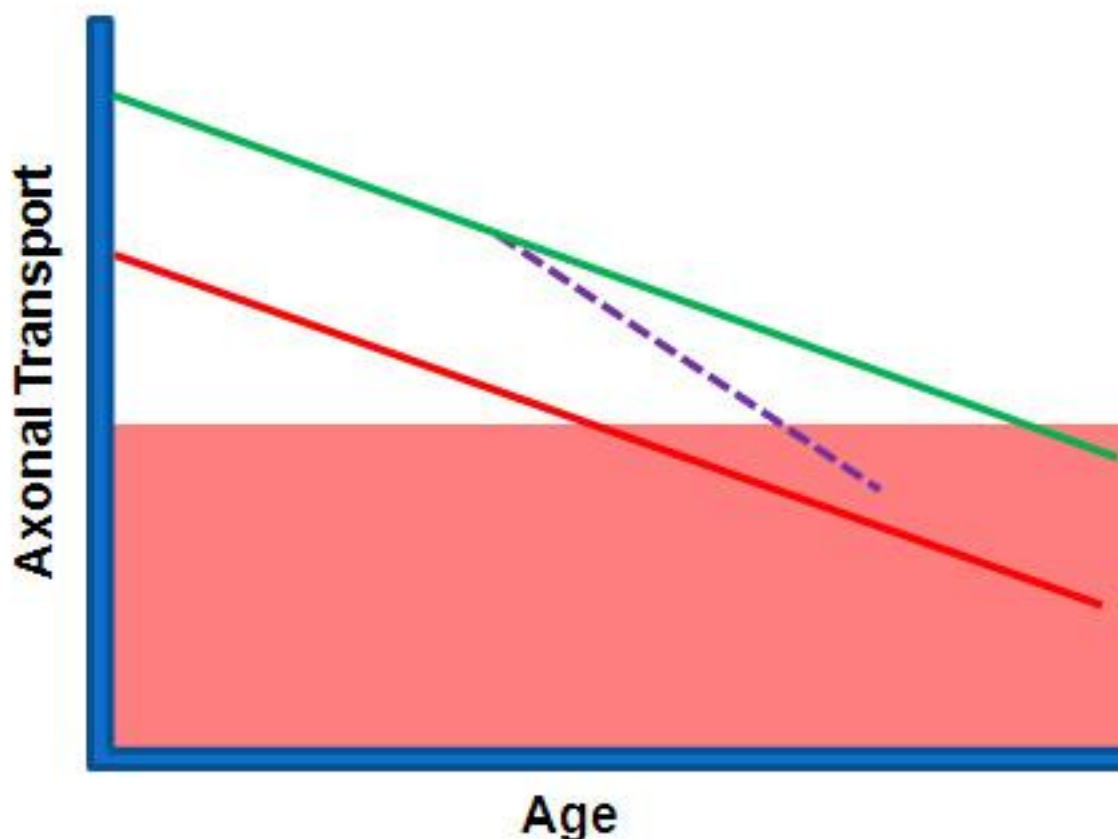


Figure 24. Model of decline in fast axonal transport with age.

The Y-axis represents the overall efficiency of FAT, and the x-axis represents an individual's age. The green line represents normal aging. People are born with a surplus of efficiency of FAT, and there is normal decline in this efficiency as they age. This decline of FAT with age takes over 100 years for the efficiency to go below the threshold for neurons to function properly (pink shaded area). With a normal life expectancy of 70 to 90 years, very few people live long enough to reach this stage. However, when somebody is born with compromised FAT due to mutations in proteins such as Kinesin-1 or PS-1, or with dysregulation in phosphotransferase activity (red line). In combination with the normal rate of decline in FAT efficiency, he/she reaches the threshold at much earlier age and this will manifest as neuropathy due to impaired FAT. The rate of decline in FAT efficiency can also change (purple dashed line) leading to the development of neurodegenerative disease (i.e. sporadic AD).

well above the threshold and it would be beyond the normal lifespan when it reaches the threshold. However, any insults that lead to reduction of transport, such as stroke or traumatic brain injury, can accelerate the decline of FAT with aging (Fig. 24 purple dashed line). The presence of mutant proteins that reduce FAT lowers the initial FAT capabilities and an individual will reach the threshold earlier (Fig. 24 red line). Several factors might affect the rate of decline of FAT with aging. Integrity of regulatory mechanisms (i.e. kinase activities) is one of the factors.

Dysregulation of CK2 and GSK3 in AD have been studied extensively. Activity and localization of CK2 plays an important role during early neural development. For example, localized activity of CK2 at the axon initial segment during neuritogenesis is essential for formation of the AIS (Sanchez-Ponce et al., 2011). The AIS is where the action potential is initiated (Clark et al., 2009), and proper formation and maturation of the AIS is critical for neuronal functions. Moreover, there is developmentally regulated expression of CK2 α and CK2 α' subunits during post-natal neocortical maturation in rat brain (Diaz-Nido et al., 1994). GSK3 is also involved in neural development. GSK3 is found to be localized in growth cone in rat primary cortical neurons (Morfini et al., 2002b), and partial inhibition of GSK3 in neuron favored neurite outgrowth (Munoz-Montano et al., 1999). This is an example of a physiological role for a kinase to regulate kinesin-1 based FAT. At the growth cone where large amount of materials are needed, GSK3 may promote delivery of cargoes. In agreement with this developmental role of GSK3, kinesin-1 is also shown to be enriched at growth cone (Morfini et al., 2001). In addition partially inhibiting GSK3 activity prevents untimely release of the cargoes. In high density culture of primary mouse cortical neurons, cells go through extensive morphological transformation from stage 1; formation of lamellipodia to stage 5: maturation of

axonal and dendritic arbors (Dotti et al., 1988) during the first 5 to 6 days. We attempted to detect the changes in CK2 and GSK3 activities associated with neural development and maturation. In vitro kinase assays of permeabilized primary mouse cortical neurons at DIV1, 2, and 5 revealed CK2 and GSK3 activities do not change during first 5 days in culture. This could be, again, due to activation of specific pools of kinase. Identification of different pools of the kinase and development of methods to assess their activity separately will elucidate developmental regulation of kinases.

Another factor that can alter the rate of decline of FAT with aging is availability of motor proteins. Distribution of KHC isoforms is tissue specific; kinesin-1A is expressed exclusively in the nerve tissues, whereas kinesin-1B is expressed in almost all tissues except for liver (Yimei You, unpublished data). There is a differential expression pattern of KHC isoforms among different parts of brain. In olfactory bulb, kinesin-1A is the most abundant KHC isoform, but kinesin-1B is the primary KHC isoform in cortex of adult mice (Yimei You, unpublished data). Also expression of KHC examined with pan-KHC antibody, H2 was developmentally regulated (Morfini et al., 2001). Tissue lysate from cortex, hippocampus, and cerebellum were collected from C57BL6 mice at different age to examine the developmental expression patterns of KHC isoforms in different parts of brain. Immunoblot analysis reveals consistently higher expression levels of all KHC isoforms in all three area of brain examined in early post-natal period and drop in protein levels in older animals. This is consistent with a previous study using H2 antibody which revealed peak of KHC expression at around post-natal week 1 and 2. In mouse cerebellum, all major events in neural development, proliferation, cell migration, differentiation, dendrogenesis, axogenesis, synaptogenesis and myelination occur in first few weeks after birth (CDT-DB, RIKEN, JAPAN www.cdtb.neuronf.jp/CDT/About.jsp). It is not surprising that

neurons respond to demands for elevated transport of cargoes during this time with increased expression of kinesin-1. Reduced availability of kinesin at older age suggests decline of FAT capabilities with age may be due to lower levels of motor protein, and may make neurons more vulnerable for insult to any component of FAT. Further studies should be undertaken to determine mRNA levels of KHC isoforms and profile of other proteins involved in FAT such as KLC and cDyn component.

CHAPTER VI

CONCLUSIONS

Alzheimer's disease is the most common adult-onset neurodegenerative disease that affects over 5 million people in the US. Anyone can be struck by AD since the greatest risk factor is old age. The cognitive deficit is the major symptoms of AD. It causes not only physical and emotional stress but also great financial burden on the caregiver, usually a family member of the patient. Although AD has been extensively researched since its discovery in 1907, currently there is no therapy that can halt or even reverse the progression of the disease.

Because of their highly polarized morphology, neurons are exceptionally dependent on the fast axonal transport system. In some neurons, motor proteins, kinesin-1 and cDyn, transport cargoes for over a meter in length. With the long distance they have to travel, motor proteins are required to have high processivity, and precise targeting mechanisms to ensure cargoes are delivered to the right locations (i.e. sodium channels to nodes of Ranvier, and synaptic proteins to the synapse) at right time. Activities of phosphotransferases such as kinases and phosphatases regulate FAT. Since there is no or very limited protein synthesis in the axon, disruption of FAT leads to synaptic failure followed by apoptosis in dying back fashion.

An appreciation of the complex regulatory mechanisms of FAT by phosphotransferases has led us to hypothesize that FAT is impaired in AD. And this impairment is mediated by protein kinase CK2. Here we present novel mechanism of pathogenesis of AD that $\alpha\text{A}\beta$ and mutant PS1-induced impairment of FAT is mediated by activation of CK2. The activation of CK2 inhibits KHC binding to MT, and disrupts interaction of DHC and DIC. Our model of

pathogenesis of AD (Figure 25) takes the fact AD is a multi-factorial disease involving all three pathological characteristics into account. Impaired FAT caused by dysregulation of regulatory kinase activities leads to dying back neuropathy of selective neuronal populations. First sign of dying back neuropathy is a synaptic dysfunction which leads to loss of synaptic connections, and followed by retraction of affected axons. Apoptotic neuronal death occurs at a very late stage of this process.

Our current study identified CK2 as a novel target for therapeutic intervention of AD. However, devising an effective therapy requires more extensive research to identify the specific pool of CK2 and nature of its activation by pathological proteins.

Figure 25

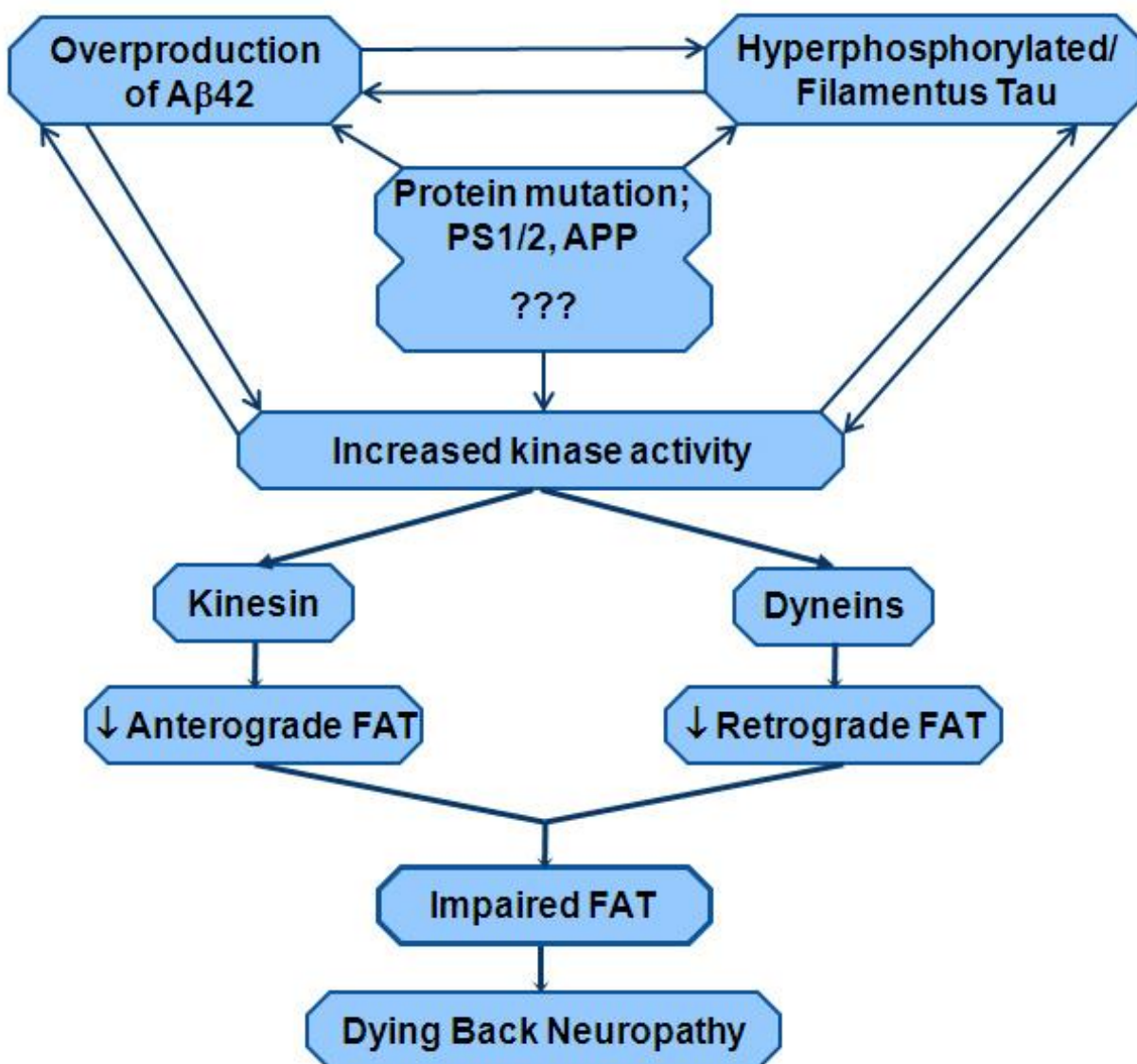


Figure 25. Proposed model of pathogenesis of Alzheimer's disease.

In the pathogenesis of Alzheimer's disease, multiple factors are involved. Overproduction of A β 42, hyperphosphorylation and filament formation of tau, and increased kinase activities are all caused by protein mutations in PS1, PS2, or APP, or by some yet to be elucidated mechanisms such as the presence of ApoE4 allele or environmental factors. Among those factors, the increased kinase activity of CK2 and GSK3 β leads to increased phosphorylation of the motor proteins, Kinesin and Dynein. Phosphorylation of Kinesin and Dynein by CK2 leads to impaired FAT through compromised motor protein binding to the MT. Impaired FAT eventually leads to dying back neuropathy of affected neurons, and manifests as AD.

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B.A., University of Illinois at Chicago, Chicago, Illinois 2003
Psychology

Teaching Experience

2006 (January – May) Teaching Assistant, Neuroanatomy, University of Illinois at Chicago

Other Experience and Professional Memberships

2009- Member, Society for Neuroscience
2008- Member, American Association for the Advancement of Science
2008- Member, American Society for Neurochemistry
2007 – 2011 Organizer, Chicago Brain Bee Competition
2007 – 2009 Member, Senate Executive Committee, University of Illinois at Chicago
2006 – 2007 Departmental Representative to the Graduate Student Council
2005 – 2006 Departmental Alternate Representative to the Graduate Student Council

Honors and awards

2011 Young Investigator Education Enhancement (YIEE) Travel Award to attend the annual meeting for the American Society for Neurochemistry
2009 – 2012 University of Illinois at Chicago Graduate Council Travel Award
2009 – 2012 University of Illinois at Chicago Graduate College Presenter's Award
2007 University of Illinois at Chicago Neuroscience Graduate Student Symposium, 1st place
2007 Chicago Chapter of Society for Neuroscience Annual Conference, Graduate Student Symposium, 2nd place

Publications

Pigino G, Morfini G, **Atagi Y**, Deshpande A, Yu C, Jungbauer L, LaDu M, Busciglio J, and Brady S. Novel pathogenic mechanism for intraneuronal amyloid beta: Disruption of fast axonal transport. *Proceedings of the National Academy of Sciences* 2009 Apr 7;106(14):5907-12

Morfini GA, Burns M, Binder LI, Kanaan NM, LaPointe N, Bosco DA, Brown RH Jr, Brown H, Tiwari A, Hayward L, Edgar J, Nave KA, Garberrn J, **Atagi Y**, Song Y, Pigino G, Brady ST. Axonal transport defects in neurodegenerative diseases. *Journal of Neuroscience* 2009 Oct14;29(41):12776-86

Presentations

Atagi, Y, Pigino, G, Morfini, G, Deshpande, A, Yu, C, Jungbauer, L, LaDu, MJ, Busciglio, J, Brady, ST. Fast axonal transport and Alzheimer's Disease; the role of casein kinase 2. Poster Presentation. American Society for Neurochemistry annual meeting in Charleston, SC. March 2009

Atagi, Y, Pigino, G, Morfini, G, Deshpande, A, Yu, C, Jungbauer, L, LaDu, MJ, Busciglio, J, Brady, ST. Mechanisms for impairment of Fast Axonal Transport by Presenilin 1 mutation. Poster Presentation. Society for Neuroscience annual meeting in Chicago, IL. October 2009

Atagi, Y, Pigino, G, Morfini, G, Brady, ST. Impairment of Fast Axonal Transport in animal models of Alzheimer's Disease. Oral Presentation. American Society for Neurochemistry annual meeting in Santa Fe, NM. March 2010

Atagi, Y, Pigino, G, Morfini, G, Brady, ST. Dysregulation of GSK3 β and CK2 in Fast Axonal Transport: Implication for Alzheimer's Disease. Poster Presentation. American Society for Neurochemistry annual meeting in St. Louis, MO. March 2011

Atagi, Y, Pigino, G, Morfini, G, Brady, ST. Activation of mouse endogenous CK2 leads to dissociation of motor protein from their track. Poster Presentation. American Society for Neurochemistry annual meeting in Baltimore, MD. March 2012