Regulation of the kinesin-3 motor, KIF1A, in a cellular model of Alzheimer's disease

by

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Abstract

Neurons are morphologically unique cells that rely on axonal transport for their function and viability. Amyloid-β oligomers (AβOs), a neurotoxin in Alzheimer's disease (AD), disrupt axonal transport via dysregulation of signaling cascades. I assessed the role for glycogen synthase kinase 3β (GSK3β), a kinase implicated in AD, in the direct regulation of the kinesin KIF1A. Inhibition of GSK3β prevented transport defects in AβO-treated primary neurons, and co-immunoprecipitation studies confirmed an interaction between KIF1A and GSK3β. Mass spectrometry on KIF1A isolated from AD transgenic mouse brain (Tg2576) showed that within a regulatory domain, Ser 402 is phosphorylated and conforms to a GSK3β recognition site. The transport of a phospho-resistant (S402A) form of KIF1A was unaffected in AβO-treated neurons whereas KIF1A (S402E) transport is severely reduced. These data suggest that AβOs impair transport via GSK3β acting directly on KIF1A. Ultimately, this work may identify novel mechanisms of KIF1A regulation in AD.

Keywords: Alzheimer's disease; axonal transport; KIF1A; Amyloid beta oligomers; glycogen synthase kinase 3β

To my parents, for their constant support, understanding, trust and love.

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List of Acronyms

AD	Alzheimer's Disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APP	Amyloid precursor protein
AβOs	Amyloid-β oligomers
α7-nAChR	α7-nicotinic acetylcholine receptor 9
BDNF	Brain derived neurotrophic factor
CDK	Cyclin-dependent kinase
CK2	Casein kinase 2
CT	computed tomography
DCVs	Dense core vesicles
ERK2	Extracellular signal-regulated kinase 2
FAT	Fast axonal transport
GSK3β	Glycogen synthase kinase 3β
HSANII	Hereditary sensory and Autonomic neuropathy type II
HSPs	Hereditary spastic paraplegias
JIP	JNK-interacting proteins
JNK	c-Jun N terminal kinase
KLC	Kinesin light chain
KO	Knockout
LTD	Long term depression
LTP	Long term potentiation
MAPs	Microtubule-associated proteins
MRI	Magnetic resonance imaging
MT	Microtubules
mGluR5	Metabotropic glutamate receptor 5
NMDAR	*N*-methyl-D-aspartate receptor
NPY	Neuropeptide Y
NTR	Neurotrophin receptor
PAD	Phosphatase-activating domain
PET	Positron emission tomography
PIP2	Phosphatidylinositol-4,5-bisphosphate

PrPc	Prion protein
SAT	Slow axonal transport
SILAC	Stable isotope labelling by amino acids in cell culture
SOD	Superoxide dismutase
SVPs	Synaptic vesicle precursors
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen activator

Chapter 1.

Introduction

Neurons are morphologically unique cells that typically consist of a cell body, an axon, and multiple dendrites. The cell body contains the nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus, hence, acting as the principal site for protein synthesis and post- translational modifications (Craig and Banker, 1994; Squire et al., 2008). The axon and dendrites are specialized structures for communication. Dendrites are short, tapering, branched projections that receive and decode electrical signals (the action potential), whereas the axon is a long, thin projection that maintains an even caliber that typically conducts electrical impulses away from the cell body. Near its end, the axon divides into small branches making synaptic connections with targets such as other neurons or muscles.

As axons are largely devoid of biosynthetic machinery, axonal transport is crucial for a neuron's function and viability. The main players in axonal transport are microtubule (MT) tracks and molecular motors. Molecular motors transport cargoes like proteins, mitochondria, neurotrophic factors and lipids. Kinesin and dynein are microtubule-based molecular motors and carry cargo from and to cell body, respectively. Axonal transport defects lead to synaptic loss and cell death, which are features of neurodegenerative diseases including Alzheimer's disease (AD; Millecamps and Julien 2013; Wang et al., 2015).

In the first half of the Introduction I provide a brief overview of AD and how a protein implicated in AD, amyloid beta, plays a role in triggering axonal transport defects. I also discuss the role of the microtubule-associated protein, tau, in association with axonal transport defects and AD. In the second half of the chapter, I introduce KIF1A, its pivotal

role in synaptogenesis, and role of KIF1A and its cargoes in neurodegeneration. Lastly, I outline my research objective and hypothesis.

1.1. Alzheimer's disease

Alzheimer's disease (AD), the most common cause of dementia in aged humans, is a progressive neurodegenerative disorder characterized by dementia and impairment in behavior, language, and visuospatial skills (Stokin and Goldstein, 2006; Trushina and Mielke, 2013). Agitation, poor judgement, withdrawal, language disturbance, hallucinations and confusion are other common symptoms associated with AD (Bird, 2014). One of the major risk factors for AD is age and after 65 years of age the possibility of developing the disease doubles every 5 years (Barranco-Quintana et al., 2005; Querfurth and LaFerla, 2010). Nearly 44 million people, over the age of 65, worldwide have AD and the diagnosis disclosure rate of AD is 1 in 4 patients only (Alzheimer's association, 2015). Presently, AD ranks as the sixth leading cause of the death in the United States with the addition of one patient every 67 seconds. Unfortunately, 5.3 million Americans and 750,000 Canadians are affected by AD and with no cure available. This number is expected to double by 2030 and more than triple by 2050. According to World Health Organization (WHO), the estimated global cost of AD is $605 billion (Information about Alzheimer's and related dementias, 2015). Unless something is done in this regard AD will take a big toll on health care (Alzheimer's association, 2015).

Alzheimer's disease was first reported in 1906 by Alois Alzheimer. Alzheimer described his patient Auguste D. in which she had suffered from memory loss, aggressiveness, and progressive confusion. Alzheimer carried out the autopsy on Auguste D.'s brain upon her death where histopathological analysis showed the presence of neuropathological lesions that are now known as neurofibrillary tangles and senile plaques (Hippius and Neundörfer, 2003). The neurofibrillary tangles are composed of paired helical filaments (PHFs) that are intracellular aggregates of hyperphosphorylated tau protein (De Vos et al., 2008). In AD, tau loses its normal function of associating and stabilizing microtubules and its aberrant phosphorylation leads to neurofibrillary tangles accumulating within neuronal cells (Avila et al., 2004). The other hallmark of AD is amyloid plaques that are comprised of extracellular aggregates of amyloid-β (Aβ) peptide. The processing of

amyloid precursor protein (APP) by the action of enzymes β-and γ-secretase results into the production of these Aβ peptides (Figure 1-1; Querfurth and LaFerla, 2010; O'Brien and Wong, 2011).

Despite of all the research to date, the only definitive way of diagnosing the disease is the post-mortem detection of the neuropathological markers of AD. Several different tests and tools have been developed in clinical diagnosis of AD which include neuropsychological tests to confirm that memory loss is not due to some other problem. Brain scans like computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET) can aid in diagnosing AD by eliminating the other possible causes of dementia, but the disease can only be confirmed after the patient's death by brain tissue autopsy (Langbart, 2002).

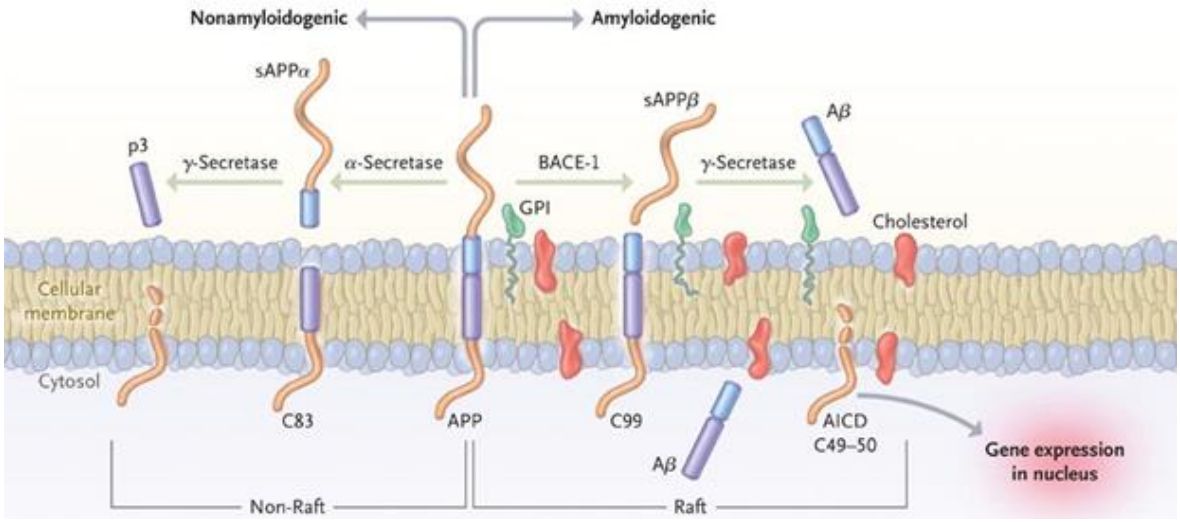


Figure 1-1: Processing of the Amyloid Precursor Protein (APP).
Nonamyloidogenic and amyloidogenic processing of APP occurs in plasma membrane. Nonamyloidogenic processing (left side of figure) is initiated by α-secretase cleavage of APP that releases the soluble ectodomain (sAPPα) in the extracellular space and generates an 83-amino acid C-terminal fragment (C83). C83 is then cleaved by γ-secretase and extracellular p3 is released. Amyloidogenic processing (right side of figure) is initiated by β-secretase and results in release of sAPPβ and retains a 99-amino acid C-terminal membrane fragment (C99). The C99 is cleaved by γ-secretase and generates Aβ peptide. The cleavage of both C83 and C99 fragments results in release of intracellular AICD fragment (Querfurth and LaFerla, 2010).

1.2. Amyloid beta

The primary role of Aβ in AD has been widely accepted (Lacor et al., 2007; Ferreira and Klein, 2011). The amyloid plaques reported by Alois Alzheimer are the abnormal extracellular aggregates of Aβ peptide. Depending on the point of cleavage on APP three different forms of Aβ composed of 38, 40 or 42 amino acid residues are produced (Walsh and Selkoe, 2007). It is the Aβ42 present in senile plaques, as this form is insoluble and prone to oligomerize (Serrano-Pozo et al., 2011). The synthesis of Aβ is a normal process, but in some people the overproduction of Aβ40 or an increased amount of Aβ42 leads to early onset of AD (Rovelet-Lecrux et al., 2006). The term early onset of AD is used for the cases in which the patients are affected with the disease before the age of 65. This form of Alzheimer's is rare and is found in only 5-10% of the patients (Campion et al., 1999). The early onset AD is of two types: familial and non-familial. The early onset familial (hereditary) AD is associated with the mutations in APP gene and the APP processing enzymes; presenilins (PSEN1 and PSEN2) hence, altering Aβ production. However, the total number of genes mutated in AD is still to be determined (Bertram and Tanzi, 2008). There is not much known about the early onset non-familial form of AD except that it develops in people in the age range of 30 or 40 (Harvey et al., 2003). The increased accumulation of Aβ is also observed in this form of disease, but how Aβ aggregation occurs might be different from familial AD (Joshi et al., 2012). Researchers are still trying to figure out why these patients are diagnosed with the disease at such an early age. 95% of the cases of AD are sporadic with aging being the major cause and several different environmental and genetic factors playing a role in disease onset (Stozická et al., 2007; Xiao et al., 2015).

The presence of Aβ in all forms of AD led to the amyloid cascade hypothesis proposed by Hardy and Higgins in 1992. This hypothesis states that the aggregation of Aβ is the initial event that triggers the disease, inducing the formation of neurofibrillary tangles, neurodegeneration and ultimately leading to dementia (Tanzi and Bertram, 2005; Reitz, 2012). Initially, it was thought that Aβ deposited in the form of plaques is neurotoxic, but later studies showed that soluble oligomeric forms of Aβ also possess neurotoxic properties and have impact on neuronal synapses (Figure 1-2; Walsh, 2002; Citron, 2004).

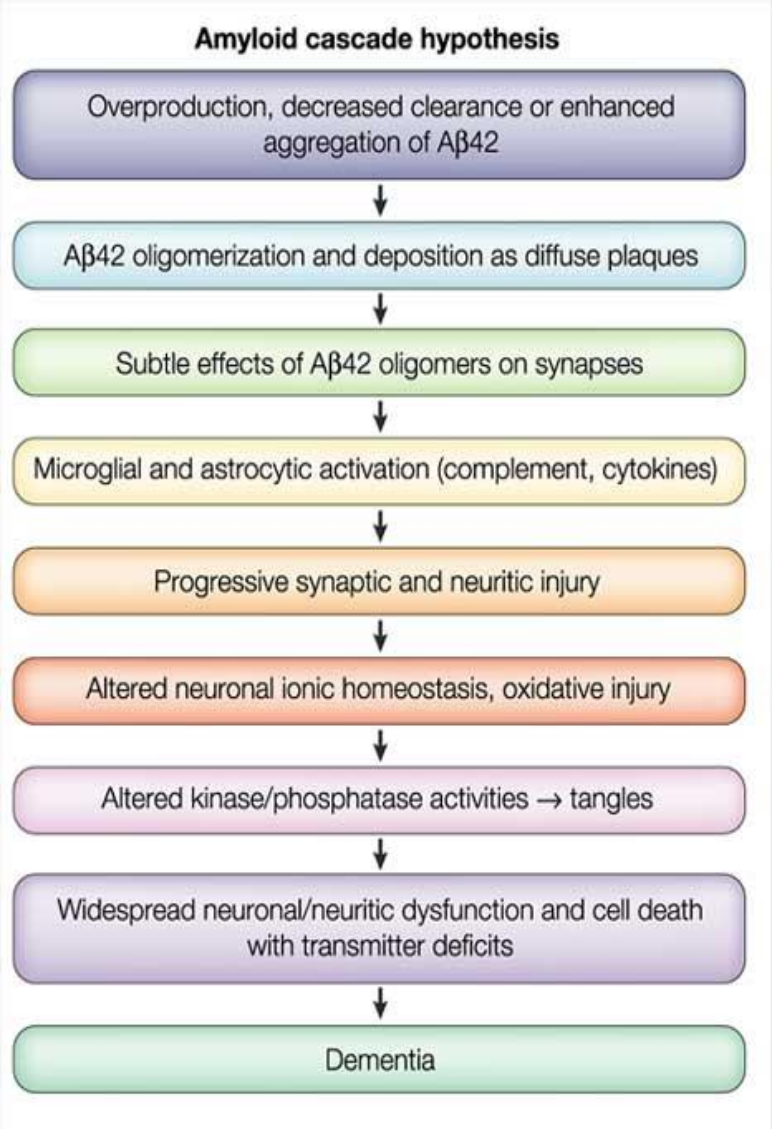


Figure 1-2: The amyloid cascade hypothesis.
Hypothetical sequence of pathogenic events triggered by Aβ that are thought to lead to AD. Notably, this is an alternative mechanism of memory loss based on the effect of soluble AβOs directly on the neuronal synapses and neurites of brain neurons (Citron, 2004).

Over the last few decades, several different studies have suggested the soluble amyloid beta oligomers (AβOs) as the neurotoxins that are more relevant to AD pathogenesis as compared to amyloid beta fibrils or plaques (Klein et al., 2001; Lublin and Gandy, 2010; Lesne et al., 2013). In fact, promoting amyloid beta fibril formation in a way that bypasses or rapidly sequesters oligomer formation is thought to be beneficial in AD (Cheng et al., 2007). This gave rise to a new hypothesis called the "AβO

hypothesis" or "oligomer cascade hypothesis" (Figure 1-3). Studies have shown that AD patients have elevated levels of AβOs as compared to control subjects (Santos et al., 2012). In a separate study on rats, these oligomers are sufficient to cause cognitive impairment (Poling et al., 2008). Hence, reiterating that oligomers cause memory loss before neuronal death (Klein et al., 2001). These oligomers lead to a number of cellular insults including the dysregulation of intracellular signaling cascades, synaptic dysfunction, and ultimately cell death (Ferreira and Klein, 2011).

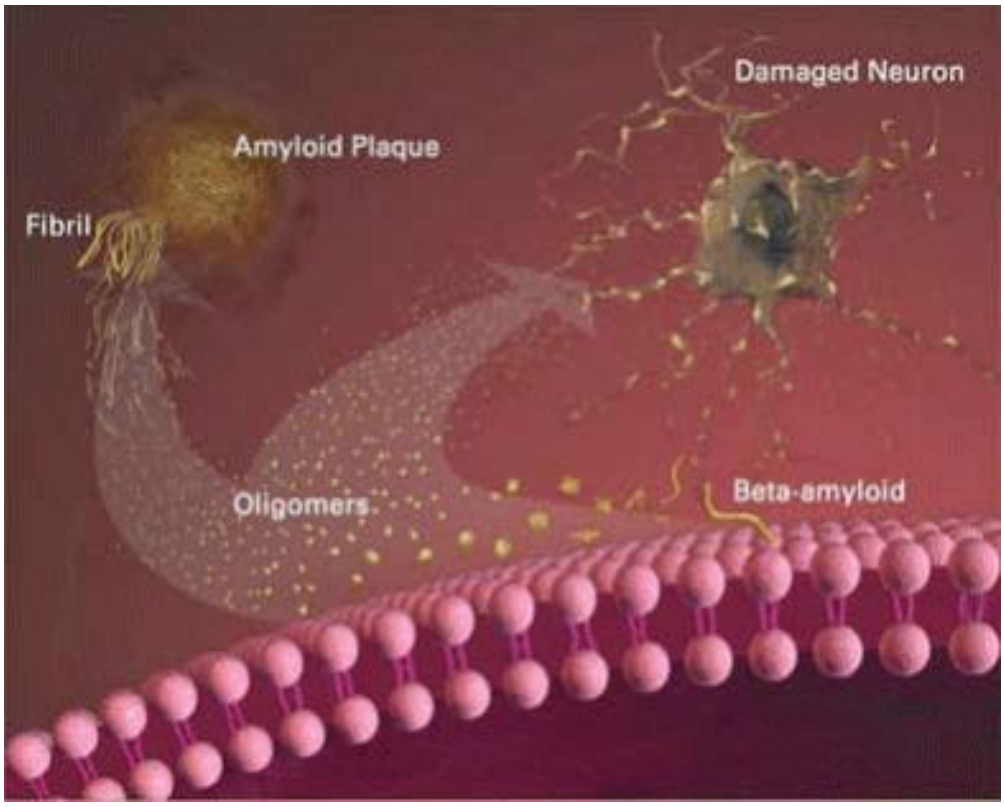


Figure 1-3: The oligomer cascade hypothesis states that the neurotoxins in AD are AβOs.

Amyloid beta oligomerizes to form AβOs that then bind and damage neurons (Right side of picture). The amyloid beta can aggregate into fibrils and lead to amyloid plaque formation, which is less toxic than AβOs (Left side of picture) (Health and Human Service Progress Report on Alzheimer's disease, 2004-2005).

AβOs bind to several different receptors on the plasma membrane and cause synaptotoxicity leading to neurodegeneration. AβOs affect the excitatory post-synaptic terminals by binding to various different receptors that includes *N*-methyl-D-aspartate

receptor (NMDAR), metabotropic glutamate receptor 5 (mGluR5), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), neurotrophin receptor and the α7-nicotinic acetylcholine receptor 9 (α7-nAChR) (Figure 1-4; Zhao et al., 2010; Dinamarca et al., 2012). The exact mechanism by which oligomers cause neurotoxicity is unknown; however, studying the receptors to which they bind can give some insight. The receptors that play an essential role in synaptic plasticity and long term potentiation (LTP) induction are NMDARs (Danysz and Parsons, 2012; Dinamarca et al., 2012). Long-term potentiation is a form of synaptic plasticity that is extensively used as an experimental model to investigate the processes of memory formation and learning in the hippocampus (Bliss and Collingridge, 1993). The NMDAR knock down study by Decker et al. showed that the NMDAR is vital for AβOs binding at the synapse (Decker et al., 2010). The AβOs directly activate NMDAR either by binding directly or close to them (De Felice et al., 2007; Texido et al., 2011). One of the physiological changes induced by AβOs is overactivation of NMDARs leading to perturbation of calcium homeostasis and inhibition of LTP (Mota et al., 2014). Furthermore, other studies demonstrate that AβOs reduce the amount of surface NMDARs by endocytosis via an α7-nAChR dependent manner, and the loss of NMDAR has also been observed in AD brain (Snyder et al., 2005; Lacor et al., 2007). The amyloid beta peptide is also observed to play a role in α7-nAChR downstream ERK2/MAPK signalling pathway that is linked with memory formation (Dineley et al., 2001). The extent of amyloid beta deposition and cholinergic neuronal degeneration in the forebrain dictates the level of learning and memory defects in AD (Shen and Wu, 2015).

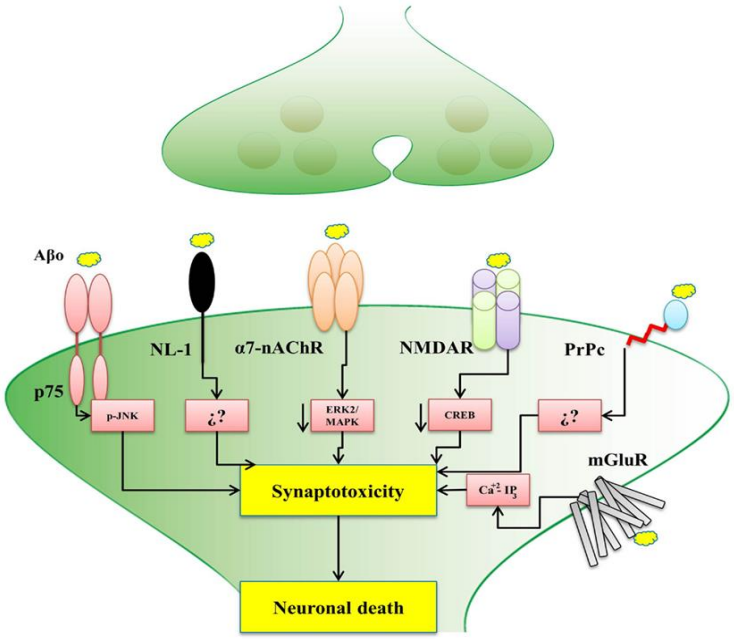


Figure 1-4: The postsynaptic receptors for Aβ Oligomers.
Aβ oligomers bind to several different postsynaptic receptors and initiate different signalling pathways that trigger synaptotoxicity, ultimately leading to neuronal death. The precise signalling pathways by which these recepors cause synaptotoxicity are well known while the mechanism by which NL -1 and the PrPc lead to neuronal death require further investigation (Dinamarca et al., 2012).

Recent findings have shown a physical interaction of AβOs with the cellular prion protein (PrPc) but the effect of this interaction on synaptotoxicity is not clear (Lauren et al., 2009; Kudo et al., 2011). AMPARs are present at excitatory synapse where AβOs bind and cause their internalization in a calcineurin-dependent manner. Experiments in cultured hippocampal neurons have shown that the pharmacological inhibition of AMPAR, primarily its subunit type 2 results in inhibition of AβOs binding on dendritic spines (Zhao et al., 2010). Cultured neurons exposed to AβOs also caused a decrease in postsynaptic density-95 (PSD-95), a scaffolding protein that plays a key role in AMPAR and NMDAR stabilization (Roselli et al., 2005). The metabotropic glutamate receptor 5 (mGluR5) is also affected by AβOs. AβOs form pathological clusters by aggregation at synaptic space and these clusters then interact with mGluR5 resulting in their abnormal accumulation. This aggregation triggers several aberrant pathological events including calcium

dysregulation, NMDAR loss and synaptic disturbance (Renner et al., 2010). Several studies support that AMPARs, NMDARs and mGluRs can play a key role in AD pathogenesis (Parameshwaran et al., 2008). Furthermore, researchers have also shown a role of the p75 neurotrophin receptor (p75 (NTR)) in neurodegeneration triggered by AβOs (Diarra et al., 2009; Knowles et al., 2009). These studies emphasize the clinical importance of AβOs in AD, making them an important target in the study of AD pathogenesis.

The effect of AβOs on the synapse suggests a basis for the neuronal connectivity loss and consequently the cognitive deficits are observed in patients with AD. A classic study by Terry and his colleagues demonstrated that the main culprit of cognitive impairment in AD is synapse loss (Terry et al., 1991). AβOs cause synapse dysfunction and degeneration by impairing synaptic plasticity. Morris and his colleagues performed an *in vivo* experiment to show that the synapse destruction in AD is caused by AβOs. They extracted AβOs from human AD brain and directly injected them in mouse hippocampus causing LTP inhibition, inducing long-term depression (LTD) and dendritic spine density loss, along with memory and learning disruption (Morris et al., 2014).

It is important to emphasize that the dendritic spine loss, synaptotoxicity, accumulation of significant amount of AβOs are well defined in AD cases and transgenic mouse models of AD (Calon et al., 2004; Gong et al., 2003; Jacobsen et al., 2006;Lacor et al., 2004; Lanz et al., 2003; Moolman et al., 2004; Spires et al., 2005). AβOs binding to postsynaptic receptors lead to the change in synapse shape and composition and could be the underlying problem behind initial cognitive impairment in the individuals suffering from AD (Lacor et al., 2007).

1.3. Microtubule based axonal transport

Neurons are morphologically unique cells that typically extend an axon and dendrites; structures specialized for communication. Axons can extend up to one meter or more, but the axon is largely devoid of biosynthetic machinery, so axonal transport is crucial for neuron function and viability. Axonal transport is a process that involves the trafficking of various cargoes from their site of synthesis to the site of use, or during

endocytosis, for example, when a trophic signal travels retrogradely to the cell body (Schwartz, 1979; Brown, 2003). Axonal transport is of two types: fast axonal transport (FAT) and slow axonal transport (SAT). FAT chiefly transports membranous organelles at the rate of ~200-400 mm/day. The proteins, polysaccharides and lipids associate with membranous organelles like Golgi-derived transport vesicles, endocytic vesicles, and lysosomes and are transported to their destination by FAT. Mitochondria are transported at comparatively slow rate of ~20-70 mm/day (Brown 2003). The transport of cytosolic protein and cytoskeletal elements like neurofilaments and microtubules are transported at the rate of ~2-8 mm/day and comprises SAT (Brown, 2000). These various cargoes are transported along microtubules in a saltatory fashion; intervals of fast movement, pauses and directional shifts in the axon (Millecamps and Julien, 2013). According to recent findings, SAT and FAT both are driven by fast motors; mitochondria, vesicles and cytoskeletal elements all move at a proportionate rate, but they differ in their duty ratio, which is time spent by cargo actually moving (Figure 1-5; Brown, 2003).

The molecular motors, kinesin and dynein are the drivers of transport along microtubules in axons and dendrites. The axons are unipolar with the plus-end (β-tubulin exposed) of the microtubule pointing outwards and minus end (α-tubulin exposed) pointing to cell center while dendrites have microtubules of mixed polarity. This polarity of microtubules promotes directionality for molecular motors as they translocate (Hirokawa and Takemura). Both kinesin and dynein are ATPases and have microtubule and cargo binding domains. Anterograde transport towards the synapse is mostly carried out by plus-end kinesin motors, while retrograde transport back towards the soma is mainly maintained by the minus-end motor dynein. Motor proteins interact with their cargo either directly, or indirectly by scaffolds or linker proteins (Figure 1-7; Karcher et al., 2002). For example, mitochondria are one of the cargoes of KIF5 and the Milton-Miro complex acts as an adaptor between them (Hirokawa et al., 2009).

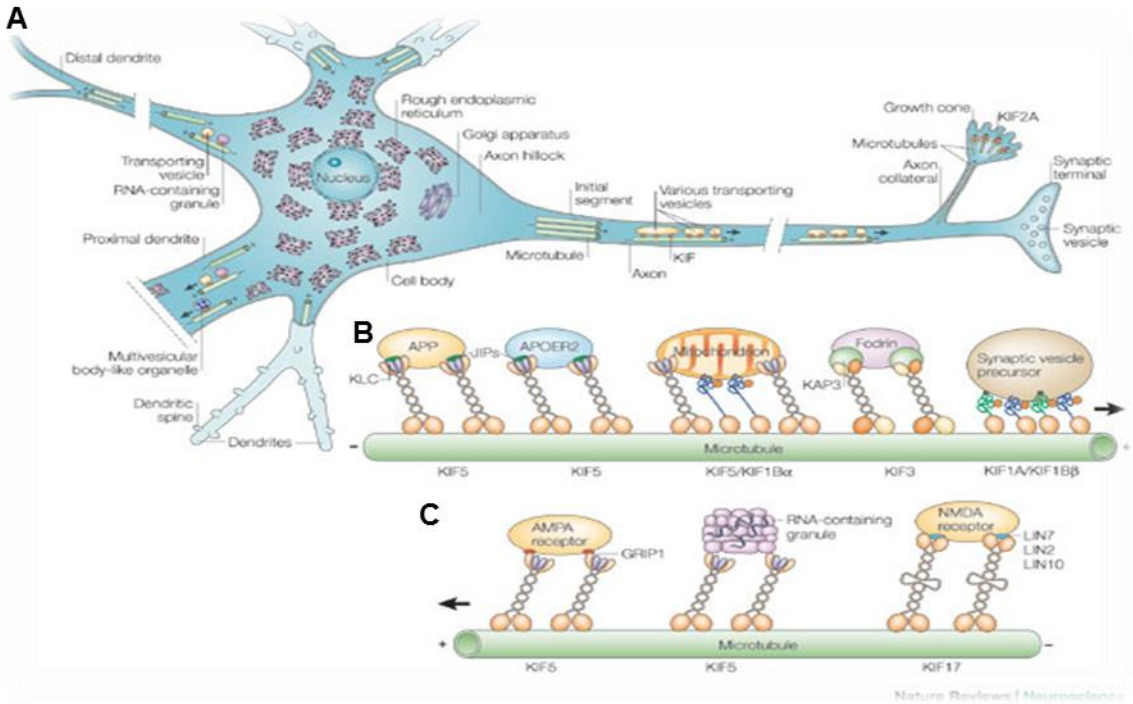


Figure 1-5: Axonal and dendritic transport.
A) A typical neuron extends a single axon (right) and multiple dendrites (left) from the cell body. B) The kinesin superfamily motor proteins are carrying vesicles containing APP, apolipoprotein E, mitochondria, fodrin and synaptic vesicles precursors in the anterograde direction in axon. C). The kinesin motor proteins transport vesicles containing AMPA receptors and NMDAR, mRNA granules in dendrites (Hirokawa and Takemura, 2005).

There are > 45 different kinesins in humans and other mammals. These proteins are classified into 14 different subfamilies on the basis of the difference in their structure (Hirokawa and Takemura, 2005). Kinesin-1 was the first fast axonal motor to be identified and is a heterotetramer composed of two heavy and two light chains (Figure 1-6). Kinesin subfamilies share the same motor domain but are significantly different in rest of their structure especially in the cargo binding domain (Goldstein, 2009; Marx et al., 2009; Miki et al., 2005).

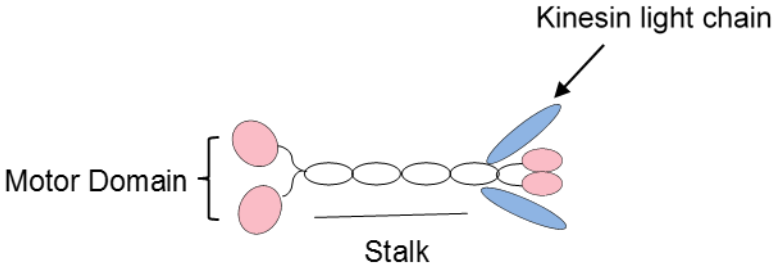


Figure 1-6: The basic schematic illustration of kinesin-1.
The motor domain of kinesin-1 consists of the microtubule binding and ATP hydrolysis sites. Kinesin-1 consists of two identical kinesin heavy chains (KHC) that dimerize to form a coiled-coil stalk region and two light chains (KLC). The tail domain is involved in cargo binding.

In its inactive state kinesin-1 is folded and immobile, but cargo binding activates it. Due to the difference in the cargo-binding domain, the kinesin subfamilies vary in their cargo specificity (Chevalier-Larsen and Holzbaur, 2006). Kinesins bind to their cargo indirectly by scaffolding or adaptor proteins or they can interact with their cargo directly with their tail region or via their light chain (Figure 1-7). Dyneins, on the other hand, are composed of two heavy chains, two light chains, two intermediate light chains and two light intermediate chains. The mobility of dynein on microtubules is regulated by its direct interaction with the multisubunit protein complex called dynactin (Figure 1-8; Schroer, 2004). Several studies suggest that kinesin and dynein motor activity is coordinated and these opposing motors interact directly, hence, influencing each other's activity (Martin et al., 1999, Gross et al., 2002; Deacon et al., 2003; Ligon et al., 2004; Kural et al., 2005). Protein phosphorylation also plays a part in regulating motor proteins activity and it involves kinases like glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (CDK-5), and c-Jun N-terminal kinase (JNK) (Cavalli et al., 2005; Morfini et al., 2009; Fu and Holzbaur, 2013; See section 1.6)

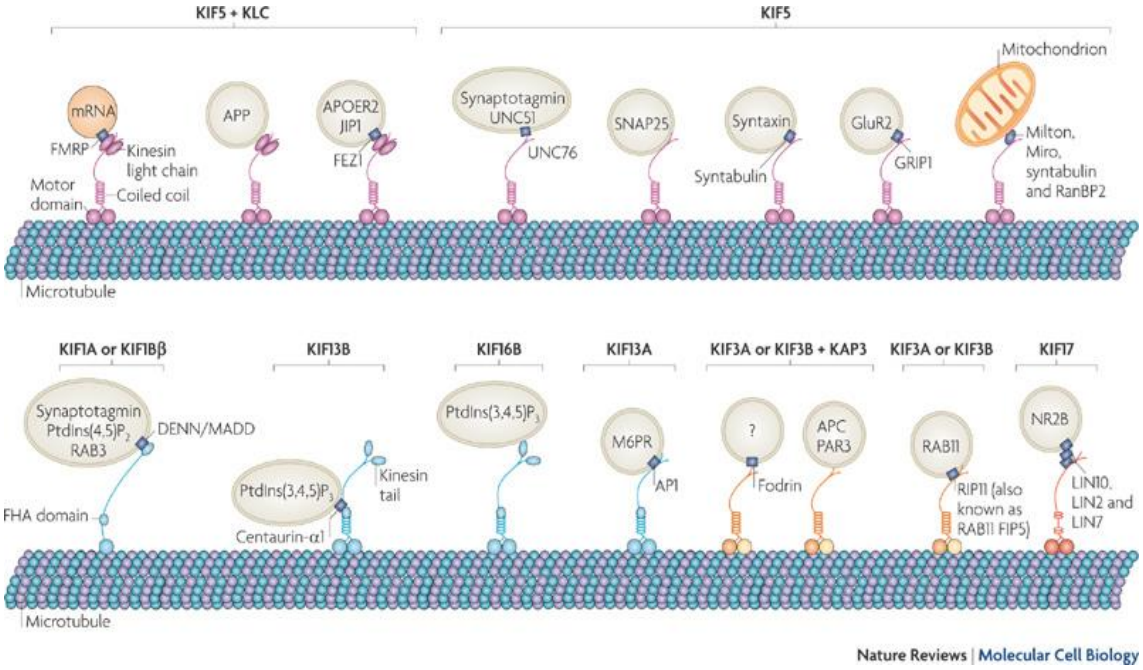


Figure 1-7: The association of kinesin motors with their cargoes via adaptor proteins or their light chain.

The kinesin-1 family motor KIF5 bind to its cargo either through or independent of its light chain. The kinesin-3 family motors, KIF1A and KIF1Bβ use the adaptor protein DENN/MADD for synaptic vesicle precursor transport. KIF13A and KIF13B also belong to kinesin-3 family motors and use adaptor proteins for the transport of their cargo. The kinesin-2 family motors KIF3 and KIF17 have dual functions in cilia, flagella and the cytoplasm. The motor-cargo pairing in each case might be determined by the post-translational modifications of protein and/or differential constitution of the adaptor complex (Hirokawa et al., 2009).

There is a very limited understanding of mechanisms underlying cargo specificity and cargo-motor complexes regulation. Particularly, the factors that play a role in motor activation, cargo binding and cargo release remain to be clarified.

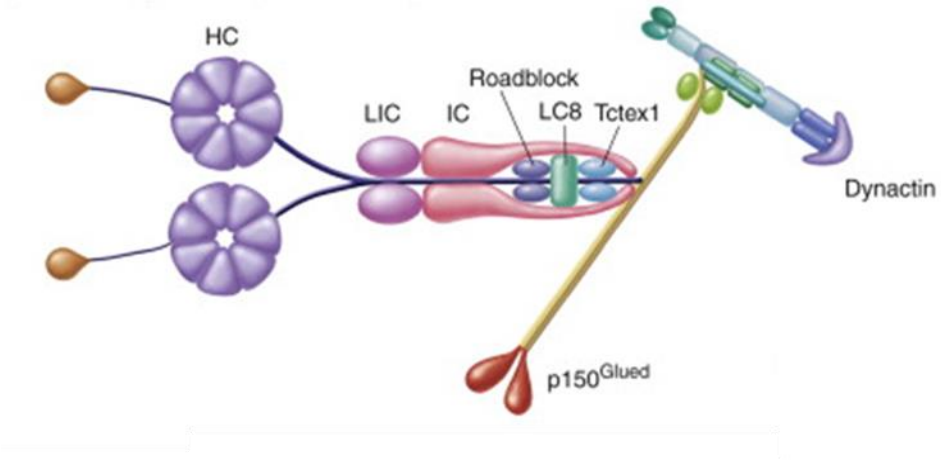


Figure 1-8: Structure of Dynein
Dynein consists of heavy chains (HC), light intermediate chains (LIC), intermediate chains (IC), and light chains (LC). To transport cargos, cytoplasmic dynein binds to the dynactin complex. Dynactin is a multi-subunit protein complex of which p150Glued protein is the largest and important for function (Hirokawa et al., 2010).

1.3.1. Microtubules structure and associated proteins

Microtubules run throughout the length of the axon and dendrites and provide cytoskeletal tracks for the movement of cellular materials. Microtubules are polymers composed of αβ-tubulin heterodimers. These heterodimers assemble protofilaments that then associate to form hollow 25 nm wide cylinders (Desai and Mitchison, 1997). Microtubules are dynamic structures that polymerize and depolymerize. For normal axonal outgrowth and growth cone formation this dynamic behaviour is required. But with maturation of the neuron, microtubules become more stable with the help of microtubule-associated proteins (MAPs) (Chevalier-Larsen and Holzbaur, 2006). MAPs are a diverse family of cytoskeletal proteins and have been divided into two broad categories: Type I including MAP1 proteins and type II including MAP2, MAP4 and tau proteins. MAP1 and MAP2 family proteins are expressed in axons and dendrites of nerve cells and help to determine neuronal structure, with MAP2 being found mostly in dendrites and tau in the axon. MAPs bind to microtubules and carry out several functions including stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in

the cell (Al-Bassam et al., 2002). MAP binding to microtubules is regulated by phosphorylation, yet when MAPs are hyperphosphorylated, which is the case in AD, hyperphosphorylated tau becomes toxic.

Tau is a microtubule stabilizing protein and is important for the maintenance of axons. Tau plays multiple physiological roles including cytoskeletal organization and stabilization, regulation of neurite outgrowth, and modulation of signalling cascades (Biernat et al., 2002; Ittner et al., 2010; Weingarten et al., 1975). Tau mutations have been reported in neurodegenerative diseases such as FTDP-17 (fronto-temporal dementia with Parkinsonism linked to chromosome 17) but there is no tau mutation associated with AD (Guo et al., 2013). In AD, tau is thought to mediate AβO toxicity and axonal transport disruption (Bloom, 2014; Ittner and Gotz, 2011). Tau hyperphosphorylation is induced by AβOs that leads to tau dissociation from the microtubules and eventual aggregation into neurofibrillary tangles (NFTs; De Felice et al., 2008). Despite the accumulation of hyperphosphorylated tau in affected neurons, the role of tau in AβO toxicity is controversial. Tau knockout neurons are resistant to Aβ-induced cell death, however other research demonstrates that NFTs persist in viable neurons possessing intact microtubules networks until late stage AD (Castellani et al., 2008; King et al, 2006). Recent studies have suggested that tau causes axonal transport disruption by initiating aberrant signalling cascade or by interacting directly with motor-cargo complexes which results in alteration of transport dynamics (Kanaan et al., 2011; LaPointe et al., 2009). However, we have previously shown that dense core vesicle (DCVs) transport disruption in cultured neurons by AβOs is independent of tau as total axonal flux was similarly and greatly reduced in the presence and absence of tau (Ramser and Gan et al., 2013). Notably, we did not observe microtubule destabilization, which suggests that microtubule-binding capacity of tau did not affect transport. In vivo data from the Nixon laboratory has also demonstrated that an increase or decrease in tau levels has no discernible effect on intracellular transport (Yuan et al., 2008). Though the role of tau is controvertial in transport defects but the experiments from our lab in tau knock out mice has shown that transport defects are independent of tau.

1.3.2. Axonal transport defects in Alzheimer's disease

Recent studies have shown that transport deficits may represent an early step in AD pathogenesis because axonal swelling and reduced axonal transport are observed before AD hallmarks such as plaque formation and extensive tau hyperphosphorylation (Stokin et al., 2005; Wang et al., 2015). Several labs (Pigino et al., 2009; Rui et al., 2006; Smith et al., 2007) have shown impaired axonal transport is a pathological feature of AD and a contributing factor to cell death during the time course of AD, but the mechanism underlying how impaired transport contributes to AD is poorly understood. The axonal transport defects in the transport of APP by KIF-5 are also related to AD progression (Gan et al., 2015; Hirokawa et al., 2010). In fact, APP mutations lead to severe axonal transport deficits resulting in neuronal damage or neurodegeneration (Roy et al., 2005). The transport deficits also arise by activation of JNK via Aβ that could cause kinesin-1 phosphorylation and hence interfere in kinesin-1 interactions with the microtubules (Bomfim et al., 2012; Wang et al., 2015). The bidirectional transport of vesicles in squid axoplasm is inhibited upon infusion of soluble AβOs as kinesin light chain (KLCs) is phosphorylated by endogenous casein kinase 2 (CK2) and results in the release of the cargo from the kinesin (Pigino et al., 2009). Our lab has shown that in hippocampal neurons axonal transport defects arise from aberrant signaling cascades initiated by AβOs. AβOs bind to the NMDA receptor leading to transport blockade in a GSK3β dependent manner (Figure 1-9; Decker et al., 2010). Furthermore, defective axonal transport is also caused by the overexpression of superoxide dismutase (SOD) or induced mutations in presenilin-1 or protein tau (Ishihara et al., 1999; Zhang et al., 2005; Lazarov et al., 2007; Massaad et al., 2010). Hence, several different mechanisms can induce axonal transport defects, which is a critical element in AD pathogenesis.

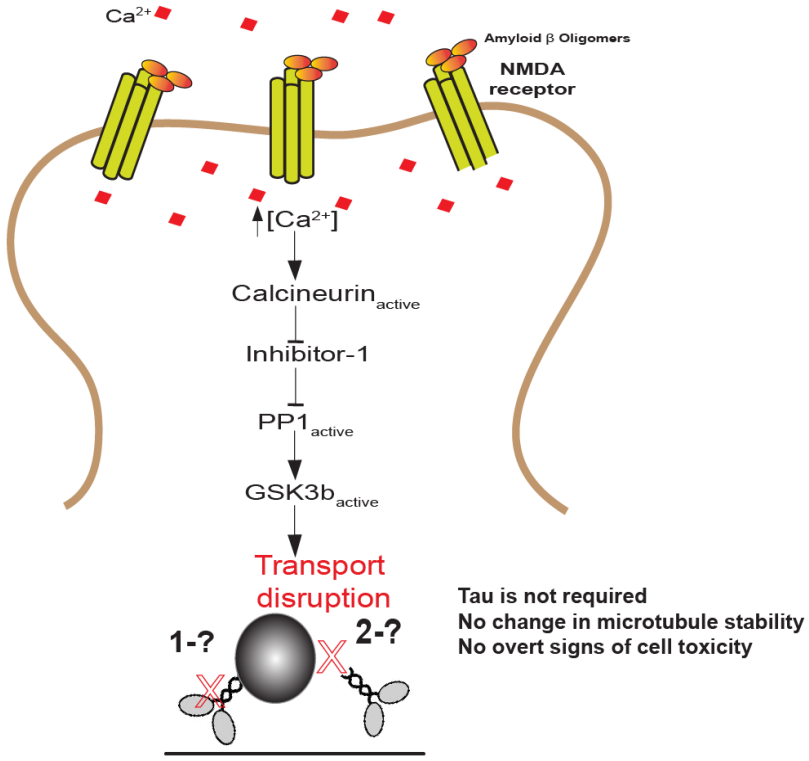


Figure 1-9: Working model for AβO-induced transport defects in an AD model. AβOs binding to NMDA receptors cause aberrant influx of Ca^{2+} ions that leads to the activation of calcineurin. Activated calcineurin, ultimately activates GSK3β by relieving inhibition of PP1. GSK3β might inhibit motor protein activity (1) and/or disrupt motor-cargo interactions (2; Adapted from Ramser and Gan et al., 2012).

1.3.3. Axonal transport, tau and Alzheimer's disease

The deficits of axonal transport are considered an early pathological event in neurodegenerative diseases like Huntington's disease, amyotrophic lateral sclerosis, and AD (Morfini et al., 2009; Trushina et al., 2004). Precisely, AD patient's brains and animal models of AD, such as, transgenic mice overexpressing human APP show involvement of axonal transport defects (Muresan et al., 2009; Salehi et al., 2006; Stokin et al., 2005). In addition manganese-enhanced MRI (MEMRI) studies in mutant APP transgenic mice and APP/PSI/tau triple transgenic mice show a reduction in axonal transport prior to neuropathological inclusions accumulation *in vivo* (Smith et al., 2007;

Kim et al., 2011). These studies provide strong evidence that axonal transport defects represent a common feature among the diverse pathologies associated with AD.

The exact mechanisms underlying axonal transport defects in AD are largely not known, but multiple pathways are likely contributors. For instance, according to some reports increasing microtubule stability causes reduction in tau-mediated axonal transport defects (Zhang et al., 2012). Some studies suggest that tau reduces axonal transport by physically interfering with the kinesin binding to microtubules (Dixit et al., 2008; Ebneth et al., 1998; Mandelkow et al., 2003) while this idea of physical blockade of kinesin by tau was not supported by other labs (La Pointe et al., 2009; McVicker et al., 2011; Morfini et al., 2009). Some recent studies show that AβOs inhibit axonal transport via multiple mechanisms including the activation of NMDAR and of specific kinases (Decker et al., 2010; Tang et al., 2012). A study conducted in our lab also shows that AβOs induce disruption of BDNF axonal transport is independent of tau and also, FAT inhibition is not accompanied by microtubule destabilization or cell death (Ramser and Gan et al., 2013). Despite the accumulation of p-tau in affected neurons its role is controversial in mediating AβO toxicity. The p-tau accumulation or dysfunction can ultimately result in neurodegenerative changes in AD (Feany and La Spada, 2003). Collectively, these findings show that p-tau ultimately accumulates and inhibits axonal transport either by directly interacting with motor-cargo complexes or by commencing abnormal signaling cascades. Though tau seems to be toxic during late stage AD, it is probably not playing role in initiating transport defects in early stage AD.

1.4. KIF1A is a primary anterograde motor

KIF1A is a mammalian homologue of UNC-104 and was first discovered in *Caenorhabditis elegans* (*C. elegans*) and is implicated in axonal transport (Hall and Hedgecock, 1991). KIF1A is a neuron-specific, highly processive motor (1.2 microns/s) staying bound to microtubules for long periods of time. This property makes KIF1A suitable for the fast anterograde transport of important cargoes like synaptic vesicle precursors (SVPs) and neuropeptide-filled DCVs which are essential for synapse maintenance and neuronal cell survival (Zahn et al., 2004: Barkus et al., 2008; Lo et al., 2011). SVPs carry synaptophysin, synaptotagmin, and Rab3A while DCVs carry various

secreted cargoes including brain derived neurotrophic factor (BDNF), tissue plasminogen activator (tPA) and neuropeptide Y (NPY) (Shin et al., 2003; Melchor et al., 2005; Tapia-Arancibia et al., 2008; Rose et al., 2009). Hence, KIF1A carries critically important cargoes required for the maintenance of the synapse (Yonekawa et al., 1998; Kondo et al., 2012).

KIF1A belongs to the kinesin-3 family that has high sequence conservation in its motor domain, fork head associated (FHA) domain and cargo binding domain (Figure 1-10; Shin et al., 2003; Miki et al., 2005). The motor domain of KIF1A is unique as it contains an extra MT binding domain specific to KIF1A called the K loop. The K loop is a highly positively charged loop and is responsible for KIF1A high processivity as it considerably increases KIF1A affinity to MTs (Kikkawa et al., 2000). The neck coil (NC) region plays a role in the KIF1A monomer to dimer transition (Tomishige et al., 2002). Moreover, KIF1A dimerization has been proposed to occur before cargo binding or on the cargo surface (Soppina et al., 2014). The regulatory region of KIF1A is composed of an FHA domain and the non-continuous small coiled-coil domains (CC1, CC2 and CC3). CC1 and CC2 play a critical part in KIF1A regulation, but the molecular mechanism underlying this regulation are not well known (Lee et al., 2004; Huo et al., 2012; Yue et al., 2013). KIF1A binds to multiple cargoes by different mechanisms. For example, KIF1A binds liprin alpha associated GRIP1 cargoes via its regulatory region and SVPs by its PH domain (Shin et al., 2003; Wozniak and Allan, 2006) but the exact mechanism of KIF1A binding to DCVs is unknown.

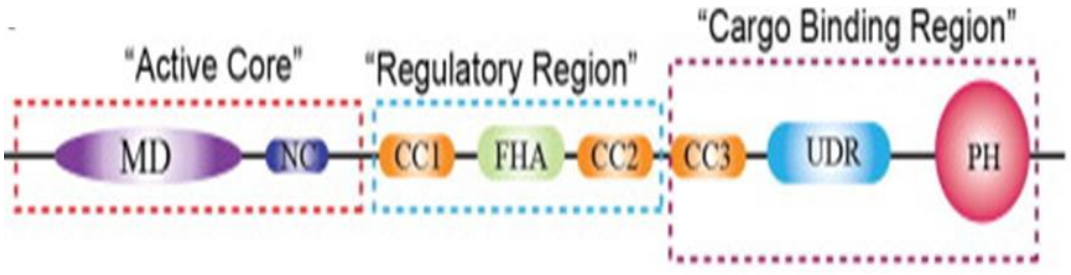


Figure 1-10: The schematic diagram of KIF1A structure.
KIF1A is composed of N-terminal motor domain and neck coil region which makes up the active core followed by CC1-FHA-CC2 domain which marks the regulatory region and a C-terminal PH domain binding cargo (Yue et al., 2013).

Several different studies have shown the motor proteins interact with their cargo in either a direct or indirect manner. A study conducted by Klopfenstein and Vale showed in *C. elegans* that the lipid binding pleckstrin homology (PH) domain of KIF1A exclusively binds to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P(2)). A point mutation in PH domain that interacts with (PI(4,5)P(2)) causes interference in motor function resulting in decreased velocity and processivity of the motor (Klopfenstein and Vale, 2004). A study in *C. elegans* also showed that subcellular distribution of KIF1A varies depending upon the type of protein complex adaptors it binds. The binding of KIF1A with UNC-16 (JIP3), DNC-1 (DCTN1/Glued) and SYD-2 (Liprin-alpha) results in localization of the motor in the soma of neurons, axonal termini and along axons, respectively (Hsu et al., 2011). Hence, cargo can modulate motor activity but phosphorylation and intramolecular folding are also shown to regulate the motor proteins binding activity (Hirokawa and Noda 2008). KIF1A can bind cargo directly (SVPs) or via adaptor proteins (liprin-alpha and GRIP1) but the role of these adaptor proteins in bidirectional transport is not clear.

KIF1A activity is modulated by the p150 subunit of dynactin complex though dynactin is associated with dynein for transport. Dynactin is a multi-subunit protein complex out of which $p150^{Glued}$ protein is the largest and is essential for function (Anderson et al., 2013). Dynactin interacts with dynein and plays its role in the bidirectional transport of DCVs (Berezuk and Schroer, 2007; Kwinter et al., 2009). Bidirectional transport can thus be achieved by coordinated activity of plus end and minus end motors, depending upon the direction of travel a particular motor can be turned “on’ or “off”, or by encouraging the cargo binding to one motor protein class over the other (Welte. 2004). Regardless of the mechanism, dynactin is known to act as a site of motor-cargo binding and coordination (Schroer, 2004). The bidirectional transport of BDNF is perturbed upon disruption of dynactin and carboxypeptidase-E interaction (Park et al., 2008). Studies in *Drosophila* have shown that KIF1A and dynein/dynactin mediated bidirectional transport is critical for driving the continuous circulation of DCVs in axons of motor neurons (Moughamian and Holzbaur, 2012; Wong et al., 2012). Despite sporadic and inefficient capture of vesicles at synaptic release sites, this mechanism yields an equal distribution of DCVs among synapses and ensures robust neurotransmission. Indeed, KIF1A mutations perturb synaptic vesicles distribution and

cause neurodegeneration in mammalian neurons (Riviere et al., 2011; Klebe et al., 2012).

The mechanisms that regulate KIF1A transport, control of cargo loading and unloading are largely unknown. Cargo binding and cargo release are suggested to be the possible mechanisms of motor regulation (Kumar et al., 2010). Several studies on KIF1A regulation have generated conflicting results. According to some models, in the inactive state KIF1A is monomeric and that activation results from concentration-driven dimerization prior to cargo binding or on the cargo surface (Klopfenstein et al., 2002; Tomishige et al., 2002). Other studies suggest that KIF1A is dimeric in inactive state and is therefore not activated by cargo-induced dimerization; rather KIF1A motors are autoinhibited, and dimeric KIF1A motors are activated by cargo binding. Because only dimeric KIF1A motors undergo ATP-dependent, superprocessive motility, failure to dimerize may prevent cargo binding and permit only diffusive movement of KIF1A monomers along the microtubule surface (Hammond et al., 2009; Soppina et al., 2014). Moreover, KIF1A dimers and monomers both can diffuse along the microtubules but the ATP dependent high processivity is only possessed by dimeric motors as monomeric motors could not move processively (Okada and Hirokawa, 1999; Hammond et al., 2009). This high processivity makes the neuron-specific KIF1A suitable for driving long-distance transport in neuronal cells.

1.4.1. KIF1A and DCV interaction

BDNF is a neuropeptide transported in DCVs and is highly expressed throughout the brain, including the hippocampus where it stimulates neuronal cell survival (Marmigère et al., 2003). BDNF plays a critical role in hippocampal synaptogenesis, learning enhancement and memory formation; therefore, it is considered a key molecule in neurodegenerative diseases (Schindowski et al., 2008; Zuccato and Cattaneo, 2009). For instance, AD progression is associated with reduced levels of BDNF (Diniz and Teixerra, 2011). The role of BDNF in neuronal physiology and disease is well established, but the motor proteins and regulatory mechanisms associated with BDNF axonal transport require better understanding (Lu et al., 2013; Scharfman and Chao, 2013). The studies from our lab and others have shown that BDNF is primarily transported by KIF1A (Barkus

et al., 2008; Lo et al., 2011). Our lab has shown that AβOs reduce BDNF transport in a GSK3β dependent mechanism, but it is unknown if the motor is directly affected (Decker et al., 2010; Ramser and Gan et al., 2011). A reduction in KIF1A motility might underlie synaptic protein loss and neuropeptide availability in AD patients, but the phosphorylation dependent mechanisms that govern KIF1A-DCV interactions and motor processitivity are unclear, and their contribution to AD has not been investigated.

1.4.2. KIF1A is a key player in the synaptogenesis

KIF1A plays a major role in the maintenance of synapse and its deficiency leads to major decline in synaptic vesicle densities and synaptogenesis (Yonekawa et al., 1998; Kondo et al., 2012). To understand the function of KIF1A Yonekawa and colleagues disrupted the KIF1A gene in mice and KIF1A knockout (KO) mice died within a day after birth due to sensory and motor disturbances. A major decrease in SVP transport was also observed which showed that KIF1A is critical for synapse formation (Yonekawa et al., 1998). Kondo et al., showed that KIF1A mediated axonal transport is enhanced by BDNF in hippocampal neurons. Moreover, increased levels of synaptophysin, cargo of KIF1A were also observed in BDNF-treated neurons. This study shows that KIF1A is upregulated by BDNF and KIF1A is critical for BDNF-induced synaptogenesis (Kondo et al., 2012). Another study in *Drosophila* showed that KIF1A not only plays a vital role in synaptic development but also important in dendritic transport for proper dendrite morphogenesis (Kern et al., 2013). Hence, KIF1A-based transport is crucial for neuronal function, maintenance and viability.

1.5. The role of KIF1A in neurodegenerative diseases

Hereditary spastic paraplegia (HSP), hereditary sensory and autonomic neuropathy type II (HSANII) are neurodegenerative diseases associated with mutations in KIF1A (Kern et al., 2013). HSP is a heterogenous group of neurodegenerative diseases and is characterized by progressive spasticity in the lower limbs. Two mutations in the KIF1A gene are responsible for autosomal recessive hereditary spastic paraplegia (Klebe et al., 2012). Another autosomal recessive disorder characterized by peripheral neurodegeneration and leading to chronic sensory loss is HSANII where a truncating

mutation in KIF1A causes disease in three different families (Riviere et al., 2011). A missense mutation in the KIF1A gene (p.Thr99Met) was reported to cause peripheral spasticity, intellectual disability and axial hypotonia in one patient while in another patient the same mutation caused the symptoms of neurological dysfunction. Hence, this p.Thr99Met mutation in the KIF1A gene is a candidate for a novel neurodegenerative syndrome (Okamoto et al., 2014). Interestingly, no KIF1A mutation has been linked to AD.

1.6. Transport disruption and cell signalling

Several kinases disrupt motor protein activity and/or cargo binding in AD. Retrograde FAT in isolated squid axoplasm is inhibited by casein kinase 2 (CK2), p38 β and JNK3; however, the molecular mechanisms by which dynein-based motility is inhibited by these kinases are unknown (Kanaan et al., 2013; Morel et al., 2012; Pigino et al., 2009). Kinesin-based transport is disrupted by glycogen synthase kinase 3β (GSK3β), casein kinase 2 (CK2), JNK, and Cyclin-dependent kinase 5 (Cdk5; Kanaan et al., 2013; Lee et al., 2011; Morfini et al., 2009; Wang et al., 2014). GSK3β is implicated in several aspects of AD (Medina and Avila, 2014). In a squid axoplasm model of AD, phosphorylation of kinesin light chain-1 (KLC1) by GSK3β signalling results in dissociation of vesicular cargo from KIF5 (Morfini et al., 2002). In *Drosophila* neurons, GSK3β acts as a negative regulator of axonal transport and reduces the number of microtubule bound motors via phosphorylation (Weaver et al., 2013). Hence, GSK3β directly impairs transport by disrupting KIF5 motor-cargo or motor-microtubule interactions (Kanaan et al., 2011; Shaw and Chang, 2013). Similarly, GSK3β has also been implicated in several studies in mammalian axonal transport disruption including work from our lab (De Furia and Shea, 2007; Decker et al., 2010; Cantuti Castelvetri et al., 2013; Ramser and Gan et al., 2013).

Although GSK3β dependent mechanisms play a vital role in inducing axonal transport defects other kinases also lead to transport deficits. The inflammatory cytokine tumor necrosis factor-alpha (TNF-α) triggers JNK phosphorylation and causes dissociation of kinesin-1 from tubulin in axons (Stagi et al., 2006). AβO-induced DCV and mitochondrial transport defects in hippocampal neurons are prevented by JNK inhibitors (Bomfim et al., 2012). Notably, the mutations in the JNK-dependent phosphorylation site S421 in JIP1 influences not only KHC activation *in vitro* but also the directionality of APP

transport in neurons (Fu and Holzbaur, 2013). CK2 activation is shown to cause transport deficits in squid axoplasm, but it is not yet clear that whether CK2 is over activated or inhibited in AD (Pigino et al., 2009; Perez et al., 2011). Cdk5 dysregulation is associated with the neurodegenerative processes of AD (Lopes et al., 2010). Cdk5 over-activation and neurofilament hyperphosphorylation perturbs their interaction with the kinesin and causes transport defects (Lee et al., 2011). The phosphorylation dependent mechanisms that cause KIF1A axonal transport defects are unknown, but these axonal transport defects are associated with neurodegeneration.

1.7. Hypothesis

I hypothesized that in a cellular model of AD, AβO-induced KIF1A transport defects are driven by GSK3β dysregulation. The main objectives of thesis were:

1- Determine if AβOs inhibit the motility of the kinesin -3 motor, KIF1A, in primary cultured hippocampal neurons

Dysregulated signalling cascades arising from AβOs disrupt cargo transport, but whether KIF1A is affected by AβOs and the role of tau on KIF1A transport dynamics is unknown.

2- Test the role of a putative GSK3β site (S402) in regulating KIF1A motility using phosphomimetic and live-cell imaging approaches

Mass spectrometry on KIF1A isolated from AD transgenic mouse brain (Tg2576) identified a phosphorylation site within the regulatory domain of KIF1A, Ser 402, which conforms to a GSK3β consensus site. I will determine the role of phosphorylation on Ser 402 on KIF1A motility.

3- Investigate a potential biochemical interaction between KIF1A and GSK3β

Experiments from our lab using GSK3β inhibitors indicate that KIF1A is a potential substrate for GSK3β (Ramser and Gan et al., 2013). I will determine if there is biochemical interaction between KIF1A and GSK3β using a heterologous expression system in conjunction with immuprecipitation and immunoblotting.

Chapter 2.

Materials and Methods

2.1. Hippocampal cell culture and expression of transgenes

Primary hippocampal neurons from E16 wild type (tau +/+) and tau knockout (tau -/-) mice were cultured as described by Kaech and Banker, 2006. Briefly, the neurons were plated onto poly-L-lysine (Sigma-Aldrich) pretreated glass coverslips and then the plated coverslips with the neurons facing down were placed into a dish containing a monolayer of astrocytes. The neurons with the astrocyte layer were kept in Neurobasal/B27 (Invitrogen) or primary neuron growth media (PNGM) (Lonza). At 10-12 days in vitro (DIV), cells were transfected using Lipofectamine 2000 (Invitrogen) with plasmids encoding soluble blue fluorescent protein (pmUba-eBFP) and KIF1A-GFP (GW1-KIF1A-eGFP; Lee et al., 2003) and shRNA sequences directed against rat KIF1AmRNA-RFP(shRNA) sequence was followed by the reverse complement (underlined), separated by a linker containing an EcoRV site. To facilitate cloning an HpaI site was incorporated at the 5' end, and an XhoI site at the 3' end. The sequence 5'-**AC**TACCTATGTGAACGGCAAGAATT**GATATC**CGTTCTTGCCGTTCACATAGGTATTTC-3' containing the rat KIF1A RNAi sequence. Prior to transfection 0.5 µM kynurenic acid (Sigma-Aldrich) was added to decrease excitotoxic damage. Cells expressed constructs for 24-36 h before imaging. The phosphorylation site point mutant of KIF1A was produced using the QuickChange II mutagenesis kit (Agilent) where serine 402 was mutated to alanine and glutamic acid. Plasmid composition was confirmed by sequencing.

2.2. REF52 and HT22 cell culture and expression of transgenes

REF52 and HT22 cells were thawed and resuspended in T-25 flasks in 5% and 10% fetal calf serum (FCS), minimum essential media (MEM) and 1 % penicillin streptomycin solution and were fed with fresh media after 24 hrs. The cells were then seeded in T-75 flask and after 48 hrs the cells were re-seeded into 6cm dishes. The cells

were transfected using Lipofectamine 2000 (Invitrogen) with KIF1A-GFP (GW1-KIF1A-eGFP; Lee et al., 2003) and HA-GSK3β S9A (Addgene). Cells expressed constructs for 24-48 h before immunoprecipitation.

2.3. AβO preparation and treatments

Soluble, full-length Aβ 1-42 peptides (American Peptide) were prepared according to the method of Lambert et al., 2007 and applied to cells at a final concentration of 500 nM for 18 h. For GSK3β inhibitor experiment, cells were treated with AβO for 18 hrs and then incubated with 5 μM Inhibitor VIII (Calbiochem) before 30 minutes of imaging.

2.4. Live imaging of KIF1A-eGFP transport

Neuronal transport was imaged using a wide-field fluorescent microscope (DMI 6000 B, Leica) equipped with a cooled CCD camera and controlled by *Metamorph,* according to Kwinter et al., 2009. The cells were sealed in the heating chamber and axon streaming videos were made at exposure time of 250 ms for 25 s. Axons were identified on the basis of morphology. To distinguish between anterograde and retrograde transport events, BFP was expressed in neurons to determine the cell body orientation. Vesicle flux, velocity, and run lengths were obtained through tracing kymographs in Metamorph, analyzed in custom software, and compiled in Excel as described in Kwinter et al., 2009.

2.5. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and blocked with 0.5% fish skin gelatin (Kwinter et al., 2009). To confirm AβO binding to dendrites, cells were stained with either mouse monoclonal NU1 anti- AβO (1:1000; Gift of W. Klein, Northwestern U.) or 11A1 (1:100; IBL) and anti-MAP2 (dendritic cytoskeletal marker; 1:2000; Millipore) and subsequently with compatible secondary antibodies (1:500; Invitrogen). For shRNA experiment, the cells were stained with mouse monoclonal KIF1A antibody (1:100; BD Biosciences) and goat anti-mouse secondary antibody (1:500; Invitrogen). For shRNA

experiment, a 10 x 4 box was drawn in the perinuclear region and then image intensity was measured by using image intensity tool in MetaMorph software.

2.6. Immunoprecipitation

Hippocampal neurons, REF52, and HT22 cells were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. 100 µg of lysate was mixed overnight at 4^0 C with a KIF1A antibody (BD Biosciences) or GSK3β antibody (Cell signaling). Samples were then combined with 40 µl of Protein A/G-agarose (Santa Cruz Biotechnology) beads and mixed at 4^0 C for 3 hr. Samples were gently pelleted and rinsed two times with ice-cold phosphate buffer saline (PBS) and one time with RIPA buffer. The immunoprecipitated proteins were resolved by SDS-PAGE and probed with anti-KIF1A (1:1000; BD Biosciences, anti-GSK3β (1:1000; Cell Signaling) and anti-HA (1:1000; Abcam).

2.7. Silver staining

The immunoprecipitated samples from mouse hippocampal neurons were resolved on SDS-PAGE and the gel was silver stained by the ProteoSilver Silver Stain kit (Sigma). The gel was fixed in ethanol and acetic acid solution for 40 min and then washed with 30% ethanol solution for 10 min. After 10 min of ultrapure water wash the gel was sensitized with sensitizer solution and washed with ultrapure water twice for 10 min again. The gel was equilibrated with silver solution for 10 min and washed with ultrapure water for 1 min. The gel was then developed for 3 to 7 min in developing solution and the development procedure was stopped by incubating the gel in ProteoSilver stop solution for 5 min. Finally, the gel was washed with 200 ml of ultrapure water for 15 min. The sample bands were cut and saved in 10 % methanol before sending them to University of Victoria mass spectrometry facility.

2.8. *in vitro* radiometric assay

For evaluating kinase activities towards their substrate a radioisotope assay was performed by Kinexus. The reaction was setup by mixing 5 µl of diluted active protein kinase (~10-50 nM final protein concentration) 5 µl of assay solution of peptide substrate and 10 µl of kinase assay buffer. The assay was initiated by the addition of 5 µl [γ-^{33}P] ATP and depending on the type of protein kinase mixture was incubated at ambient temperature for 20-40 min. The assay was terminated by spotting 10 µl of the reaction mixture onto a multiscreen phosphocellulose P81 plate and P81 plate was washed 3 times in a 1% phosphoric acid solution for ~15 min each. Trilux scintillation counter was then used to count the radioactivity on the P81 plate in the presence of scintillation fluid (Kinexus).

2.9. Analysis of flux

All videos were processed using *MetaMorph* software (Universal Imaging), which was used to generate a time-distance graph for each video with the kymograph option. Diagonal lines on each kymograph were traced and the information was analyzed by custom-made software (Kwinter et al., 2009) that calculated the values of flux, velocity and run lengths based on the calibration that at a magnification of 630X, 1 pixel = 0.160508 µm (vertical axis). Motors traveling less than 2 µm were not included in the analysis as distances this short could be accounted for by diffusion based on the formula that root-mean square displacement is where D is the diffusion coefficient (D=0.01 for KIF1A) and t is the duration of the observation (t=50 s) Kwinter et al., 2009). Runs were considered terminated if a particle remained in the same position for 4 frames (1s).

Motor flux was defined as the total distance traveled by motors standardized by the length and duration of each movie (in micron-min):

$$\text{Flux} = \frac{(\mu\text{m distance traveled})}{(\mu\text{m segment length}) \cdot (\text{min of recording})}$$

The measure of flux is an aggregate of individual moving particles in each video. Due to variation in expression levels of tagged proteins and regional differences in the neuron, e.g., proximal v. distal, flux accounts for the behavior of individual events rather than the total number of transport events.

2.10. Statistical analysis

A one-tailed student t-test, using equal or unequal variance based on F-tests, was used to determine significance between pair wise comparisons of control and experimental conditions in SPSS statistics. Significant differences between treatments were analyzed by t-tests with equal or unequal variance at a 95% confidence interval. For live imaging experiments, a minimum of 12 cells from 3 independent cultures (n=3) were analyzed.

Chapter 3.

Results

3.1. AβOs introduce KIF1A transport defects

KIF1A is the primary anterograde motor for BDNF transport, and both loss of BDNF availability and mutations in KIF1A are associated with neurodegenerative diseases (Lo et al., 2011; Okamoto et al., 2014). However, the phosphorylation-dependent mechanisms that regulate KIF1A and its potential contribution to AD have not been investigated. AβOs dysregulate signaling cascades, which in turn, may lead to perturbation of motor regulation of FAT (see Introduction). The study conducted by Kanaan et al., in isolated squid axoplasm have shown that the phosphatase-activating domain (PAD) within the N-terminal of tau exposed by AβOs induces tau hyperphosphorylation. AβOs triggers the activation of protein phosphatase 1 (PP1) and the GSK3β signaling cascade which impedes kinesin-based anterograde transport, independent of microtubule binding. The increased exposure of PAD is also one of the early pathological events in AD (Kanaan et al., 2011). However, our lab has recently shown that BDNF transport defects induced by AβOs are independent of the microtubule associated protein tau, microtubule destabilization and acute cell death (Ramser and Gan et al., 2013). In addition to studying the role of AβOs on KIF1A transport in tau-bearing neurons, I also asked if KIF1A transport disruption was independent of tau. The first aim of my project was to determine the effect of AβOs on KIF1A motility in tau +/+ and tau -/- neurons; a project initiated by former PhD student, K. Gan. To understand KIF1A transport dynamics in the presence of AβOs I expressed soluble blue fluorescent protein (BFP) and KIF1A-enhanced green fluorescent protein (eGFP) in hippocampal neurons from tau +/+ and tau -/- mice. The neurons were treated with 500 nm AβOs for 18 hrs before live imaging (Figure 3-1). Representative kymographs show the differences between transport of KIF1A in control (vehicle treated) and AβO treated neurons (Figure 3-2). The total axonal flux of KIF1A was reduced in both tau $^{+/+}$ and tau $^{-/-}$ AβO treated neurons by 68 % ± 10.98 and 57% ± 9.67, respectively (Figure 3-3 A, 3-4 A and Table 3-1). More specifically, a reduction in anterograde flux was observed in both genotypes, but retrograde flux was reduced in only tau +/+ neurons

(Figure 3-3 A). The AβOs treatment had no overall effect on KIF1A velocity in tau $^{+/+}$ and tau $^{-/-}$ neurons, in comparison to control (Figure 3-3 B and 3-4 B). KIF1A run length was unaffected by AβOs treatment in tau +/+ neurons while in tau -/- neurons anterograde run length was reduced (Figure 3-3 C and 3-4 C). Previously, our lab has imaged only KIF1A cargoes like BDNF and transport reduction may have resulted from the dissociation of the cargo from the motor. However, my results suggest that the reduction in KIF1A motility can be due to the action of AβOs on the motor itself or disrupting another motor activation mechanism, for example motor-cargo binding. Furthermore, my data also demonstrates that anterograde transport disruption of KIF1A is independent of tau while retrograde transport is reduced by the presence of tau.

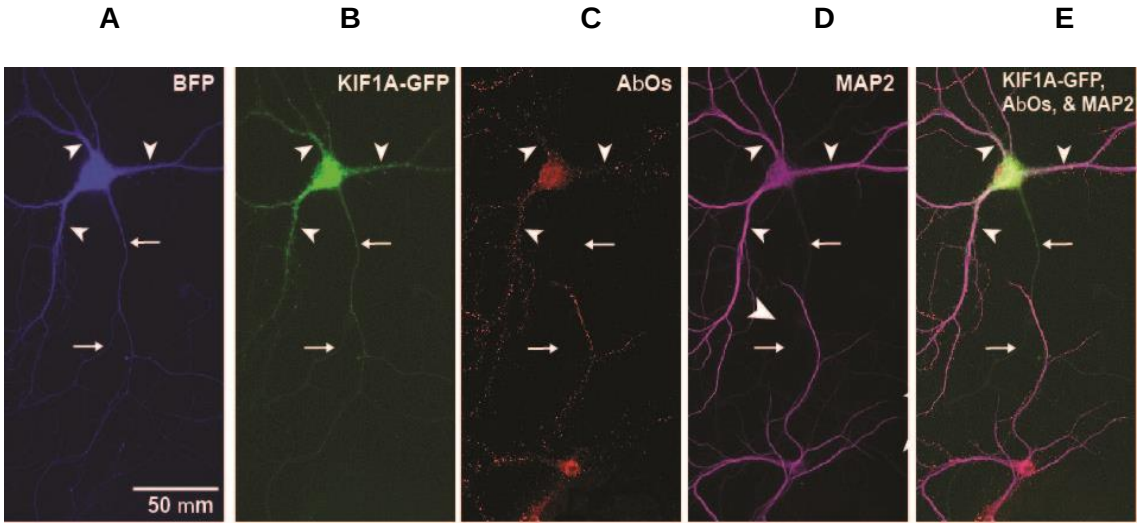


Figure 3-1: Expression of KIF1A-GFP in an AβOs treated neuron
A-E). Expression of soluble BFP and KIF1A-eGFP in an AβO-treated tau+/+ neuron (from left to right). Overlay of BFP and AβO images shows binding of AβOs exclusively to dendrites. Immunocytochemistry shows that AβOs remain oligomeric after 18 h in culture. Arrows indicate axon; arrowheads indicate dendrites.

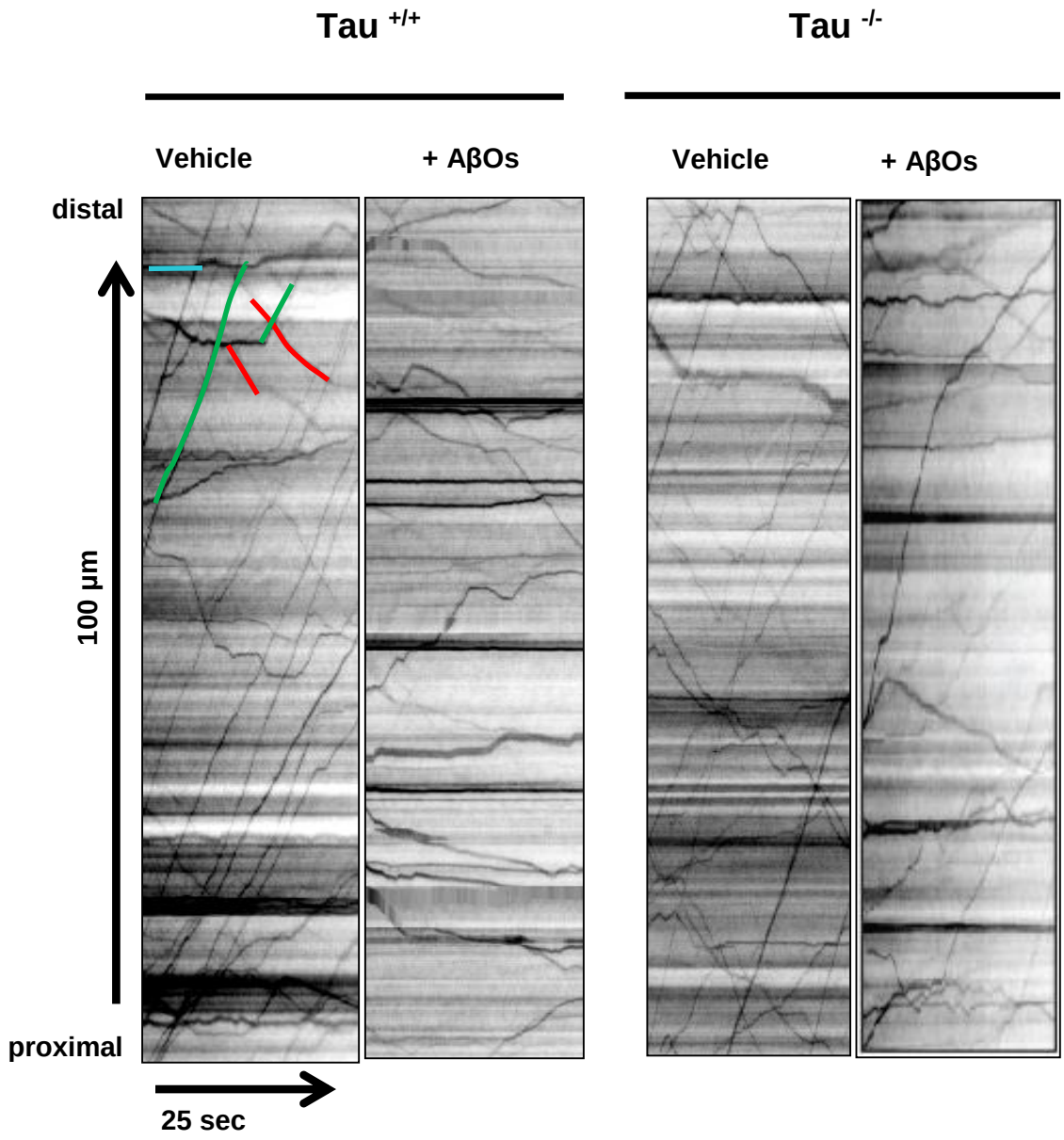
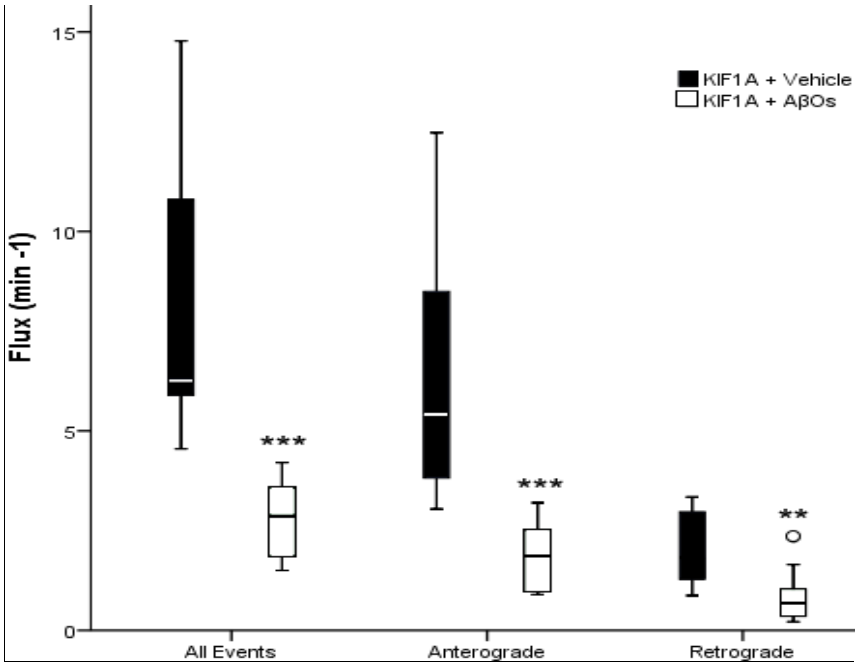
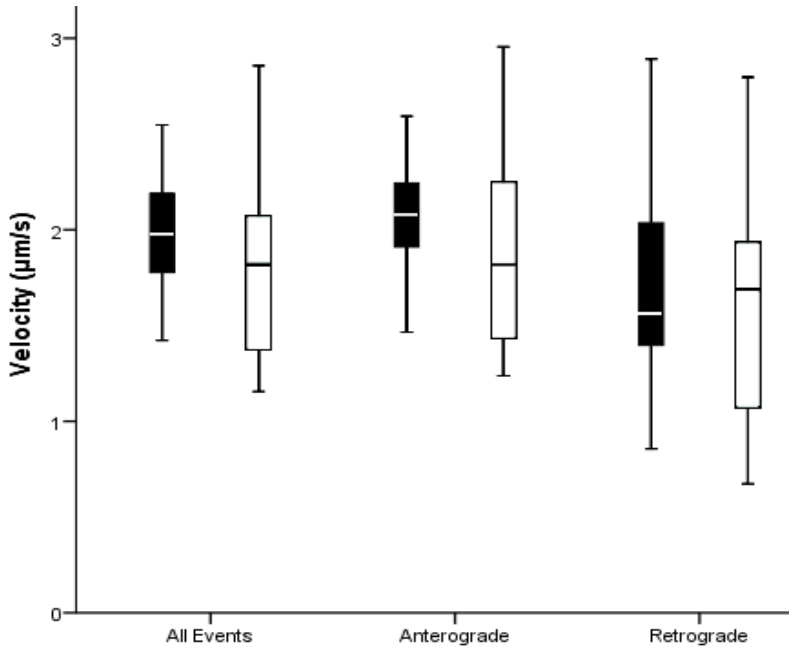


Figure 3-2: AβOs reduces KIF1A transport in tau $^{+/+}$ and tau $^{-/-}$ neurons.
Representative kymographs of KIF1A transport in control and 500 nM AβO treated tau $^{+/+}$ and tau $^{-/-}$ neurons. The neuronal cells were transfected with a KIF1A-GFP plasmid and treated with AβOs or vehicle for 18 hours before imaging. An example of the kymograph analysis is shown in the first kymograph (left). Lines with a positive slope represent anterograde transport (green); lines with a negative slope represent retrograde transport (red) and horizontal lines represent stationary motors (blue).

A) KIF1A Flux in tau $^{+/+}$ neurons



B) KIF1A velocity



C) KIF1A Run Length

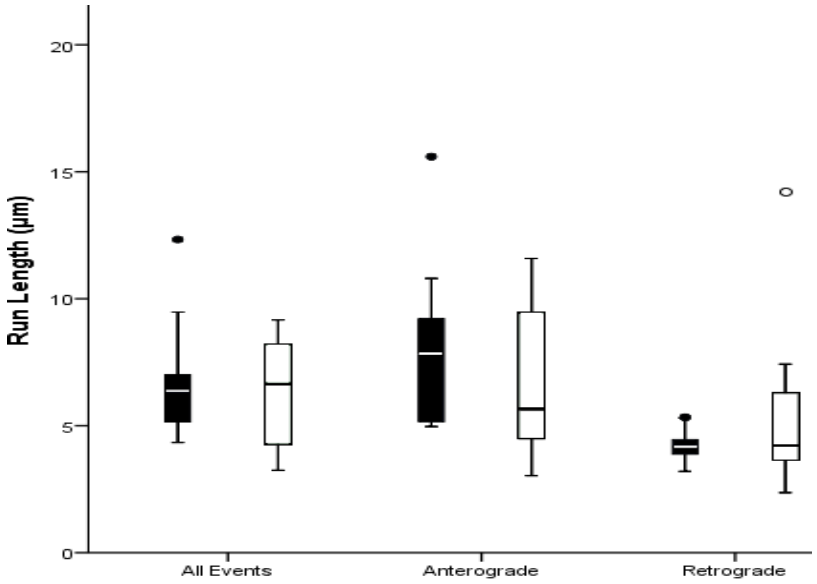
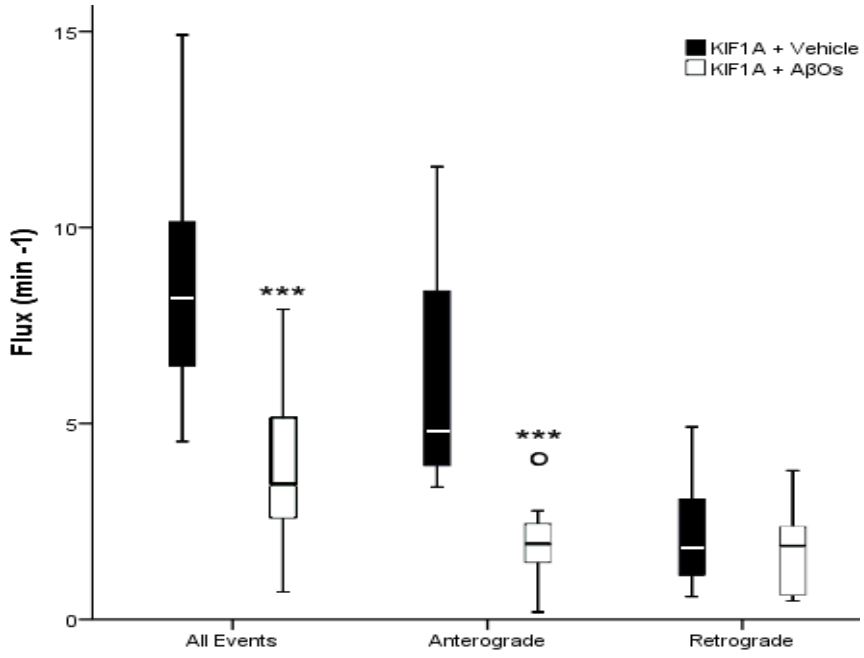


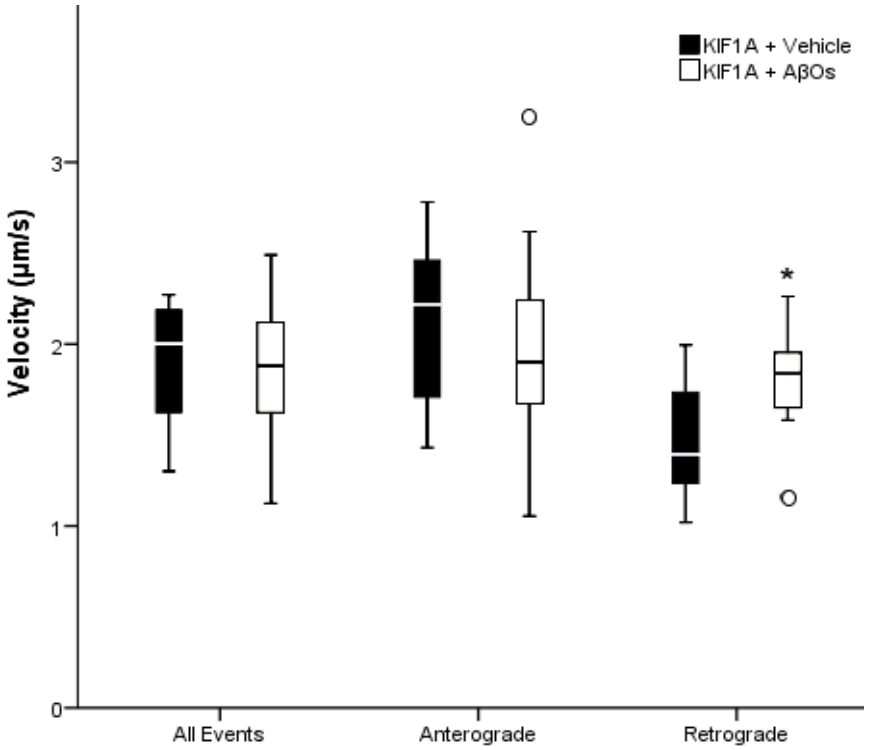
Figure 3-3 : KIF1A transport dynamics comparison in tau $^{+/+}$ neurons treated with vehicle and AβOs

Effect of AβOs treatment on KIF1A flux, velocity and run length in tau $^{+/+}$ neurons. Neurons were transfected with KIF1A-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβOs treatment decreased the bidirectional flux in comparison to vehicle control (The bottom and top of the box indicate, respectively, the first and third quartiles; the inner line indicates the median; the whiskers show the data range. Circles above box plots show the outliers). B). The average velocity of KIF1A was unaffected by AβOs treatment. C). AβOs treatment had no effect on KIF1A run length. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control.

A) KIF1A Flux in tau $^{-/-}$ neurons



B) KIF1A Velocity



C) KIF1A Run Length

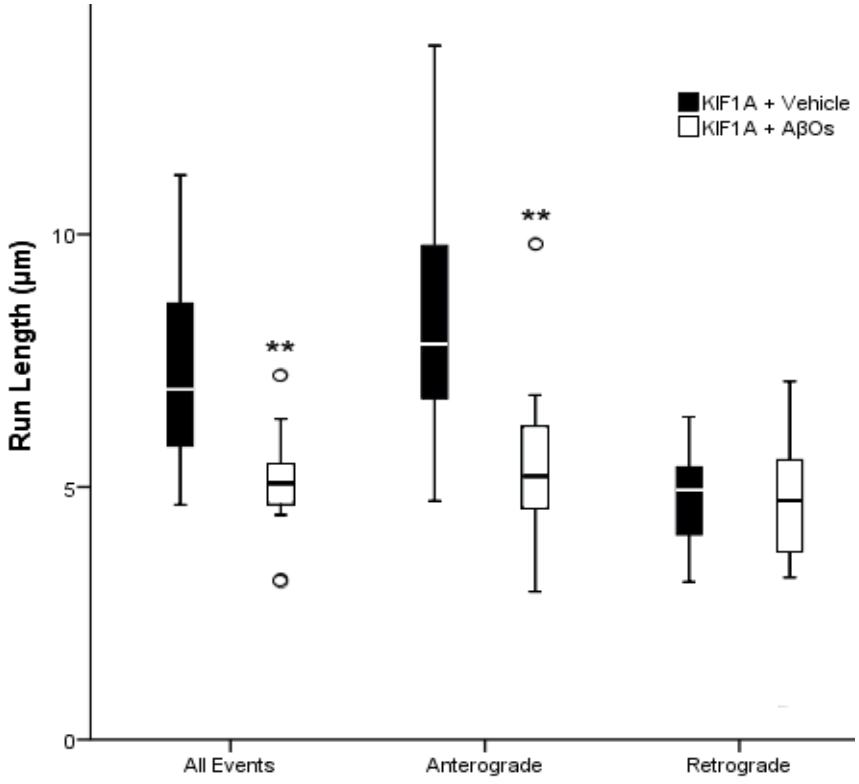


Figure 3-4: KIF1A transport dynamics comparison in tau $^{-/-}$ neurons treated with vehicle and AβOs

Effect of AβOs treatment on KIF1A flux, velocity and run length in tau $^{-/-}$ neurons. Neurons were transfected with KIF1A-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβO treatment decreased the overall and anterograde flux, but retrograde flux was unaffected in comparison to vehicle control. B). The overall average velocity of KIF1A was unaffected by AβO treatment, but retrograde velocity was decreased in AβO treated neurons in comparison to control. C). AβOs treatment caused decrease in KIF1A run length in anterograde direction but retrograde run length was unaffected in comparison to vehicle control. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control.

Table 3-1: KIF1A transport defects introduced by AβOs in hippocampal neurons.

KIF1A axonal transport in hippocampal neurons				
	Traffic Values			%
	All Events	Anterograde	Retrograde	All Events
Flux (min^{-1})				
tau $^{+/+}$, vehicle	8.56 ± 0.94	6.48 ± 0.87	2.08 ± 0.24	100.00 ± 10.98
tau $^{+/+}$, AβOs	2.79 ± 0.28***	1.88 ± 0.23***	0.90 ± 0.19**	32.59 ± 3.27***
tau $^{-/-}$, vehicle	8.58 ± 0.83	6.29 ± 0.72	2.29 ± 0.36	100.00 ± 9.67
tau $^{-/-}$, AβOs	3.70 ± 0.58 ***	1.97 ± 0.32***	1.73 ± 0.31	43.12 ± 6.75***
Velocity (µm/s)				
tau $^{+/+}$, vehicle	1.94 ± 0.08	2.06 ± 0.08	1.72 ± 0.14	100.00 ± 4.12
tau $^{+/+}$, AβOs	1.86 ± 0.15	1.96 ± 0.15	1.66 ± 0.17	95.87 ± 7.73
tau $^{-/-}$, vehicle	1.91 ± 0.08	2.11 ± 0.11	1.46 ± 0.08	100.00 ± 4.19
tau $^{-/-}$, AβOs	1.84 ± 0.10	1.92 ± 0.15	1.76 ± 0.08*	96.33 ± 5.23
Run Length (µm)				
tau $^{+/+}$, vehicle	6.58 ± 0.53	7.86 ± 0.74	4.23 ± 0.17	100.00 ± 8.05
tau $^{+/+}$, AβOs	6.36 ± 0.59	6.91 ± 0.80	5.41 ± 0.78	96.65 ± 8.96
tau $^{-/-}$, vehicle	7.20 ± 0.49	8.36 ± 0.62	4.81 ± 0.23	100.00 ± 6.80
tau $^{-/-}$, AβOs	5.07 ± 0.29**	5.43 ± 0.45**	4.68 ± 0.30	70.41 ± 4.03**

tau $^{+/+}$ KIF1A + vehicle: n = 15 kymographs (15 cells, 825 puncta)
tau $^{+/+}$ KIF1A + AβOs: n = 14 kymographs (14 cells, 300 puncta)
tau $^{-/-}$ KIF1A + vehicle: n = 15 kymographs (15 cells, 824 puncta)
tau $^{-/-}$ KIF1A + AβOs: n = 14 kymographs (14 cells, 413 puncta)

* p<0.05, when compared with KIF1A + vehicle
** p<0.01, when compared with KIF1A + vehicle
***p<0.001, when compared with KIF1A + vehicle

3.2. KIF1A dimerization domain is phosphorylated at GSK3β consensus site

I hypothesized AβO-induced neuropeptide transport disruption occurs via aberrant phosphorylation of KIF1A. In support of this idea we identified phosphorylation sites on KIF1A by tandem mass spectrometry on KIF1A isolated from 14-month old AD transgenic mouse brain (Tg2576) and compared KIF1A phosphorylation to an age-matched, wild type control. The transgenic Tg2576 mice overexpress human mutant amyloid precursor protein and are used as a model for AD (Jacobsen et al., 2006; Jung et al., 2011). E.M. Ramser isolated KIF1A from AD transgenic mouse brain (Tg2576) for tandem mass spectrometry analysis, subsequently performed by the University of Victoria Genome BC Proteomics Centre. We detected significant phosphorylation in the dimerization domain of KIF1A at Ser 402, which conforms to a GSK3β consensus site according to the Phosida (http://www.phosida.com) and Phosphonet (http://www.phosphonet.ca) database. Interestingly, this site is conserved between mouse, rat, zebra fish and human, showing that it might be critical in the regulation of KIF1A motility (Figure 3-5 A). As S402 is present in the forkhead associated domain of KIF1A it is likely to regulate its activation and motility (Figure 3-5 B).

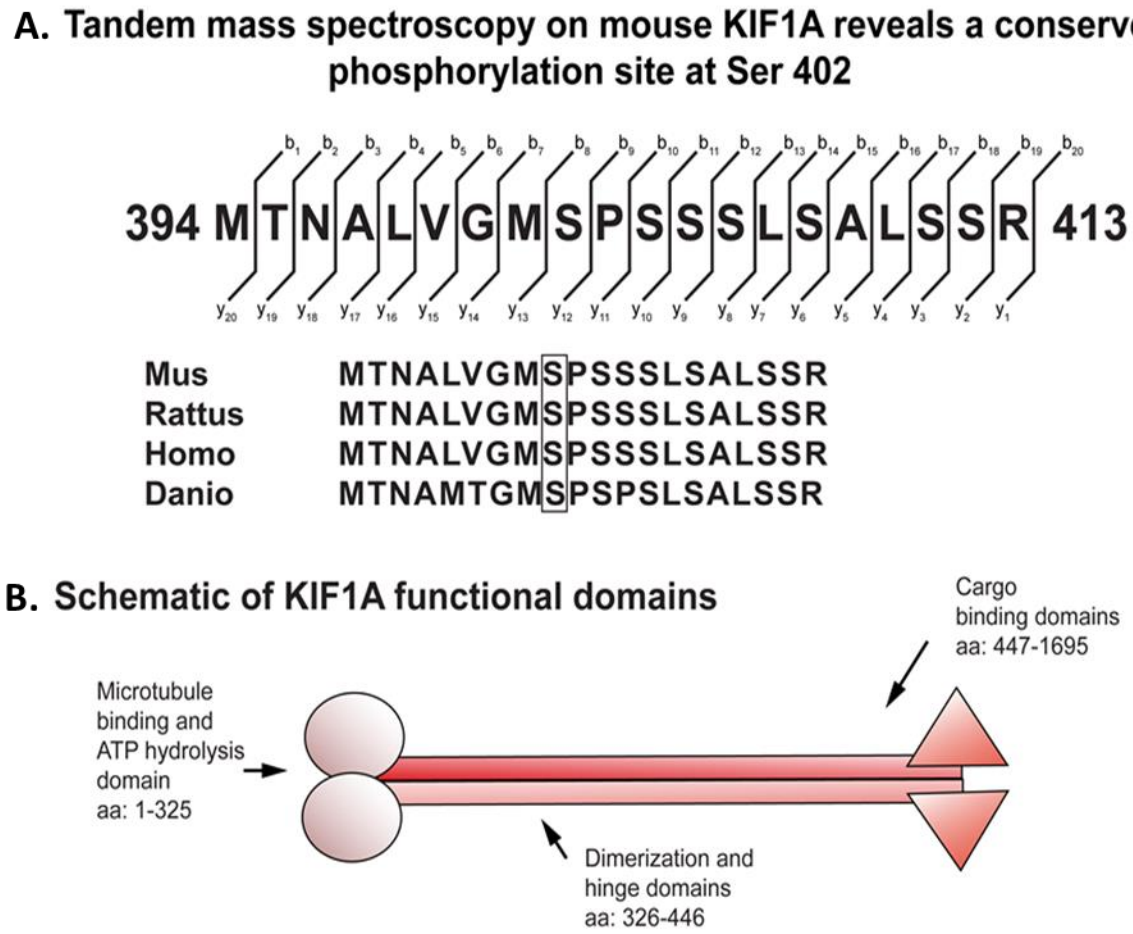


Figure 3-5: The KIF1A dimerization domain is phosphorylated at a conserved GSK3β consensus site

A) Tandem mass spectroscopy on KIF1A isolated from AD model mouse (APPswe) brain identified a phospho-peptide within the dimerization domain (amino acids 394-413). Phospho-serine 402 in this peptide conforms to a GSK3β site according to Phosida (phosida.com) and Phosphonet (kinexus.ca). Sequence alignment shows that Ser 402 is conserved between zebrafish, mouse, rat, and human. B) Basic schematic of KIF1A, a kinesin-3 family member.

3.2.1. GSK3β phosphorylates KIF1A *in vitro*

An *in vitro* phosphorylation assay was designed to assess the role of phosphorylation on Ser 402. In this radiometric assay, three different proline directed kinases (Cyclin-dependent kinase 2 (CDK2), Extracellular signal-regulated kinase 2 (ERK2) and GSK3β were tested for their ability to phosphorylate the S402 residue on KIF1A by the Kinexus substrate profiling services. For the purposes of identifying and selecting the best kinase that specifically target the KIF1A [S402] site, the differences between the counts of the wild type (WT) vs. mutant (MT) peptide is considered. In general, the effect of the alanine substitution of the KIF1A [A402] peptide revealed a significant decrease in the level of phosphorylation for all of the kinases tested when compared to the levels of phosphorylation observed by the corresponding wild type KIF1A [S402] peptide (Table 3-2).

Table 3-2: The radiometric assay data for KIF1A phosphorylation

Protein Kinase	KIF1A (S402) WT peptide (counts per min)	KIF1A (A402) MT peptide (counts per min)	Difference	% Change (MT-WT) /WT
GSK3β	51,749	1,512	50,237	-97%
CDK2	5,556	2,440	3,116	-56%
ERK2	2,631	1,957	674	-26%

The evaluation of three different proline directed kinases (CDK2, ERK2 and GSK3b) activity by radiometric assay indicates that GSK3β shows a strong preference for the KIF1A (S402) WT peptide in vitro.

The differences observed between the levels of phosphorylation between WT and MT peptide by GSK3 β kinase was noted as high as 97%. The radiometric assay indicated that GSK3β shows >10 fold preference for the KIF1A (S402) peptide compared

to the other kinases. The activity of GSK3β towards an alanine substitution at site 402 was negligible.

3.3. The role of S402 in regulating KIF1A motility

To further assess the role of phosphorylation on Ser 402, a nonphosphorylatable form and a phosphomimic of KIF1A were generated by point mutation of Ser- to-Ala and Ser-to-Glu, respectively. The transport of S402A and S402E mutations of KIF1A were analyzed in the presence and absence of AβOs to evaluate the effect of phosphorylation on KIF1A transport dynamics.

3.3.1. The phosphomutant KIF1A-S402A is motile in AβO-treated neurons

To determine the potential role of KIF1A-S402 in transport regulation, a KIF1A-S402A-eGFP was expressed in mouse tau $^{+/+}$ and tau $^{-/-}$ hippocampal neurons and compared to KIF1A-eGFP. The cells were treated with 500nM of AβOs 18 hrs prior to imaging. The representative kymographs show the differences between transport of KIF1A and KIF1A-S402A in control (vehicle treated) and AβO treated neurons (Figure 3-6). The transport data show that the KIF1A-S402A mutant is unaffected in the presence of AβOs as its flux is comparable to wild type KIF1A in tau $^{+/+}$ and tau $^{-/-}$ neurons (Figure 3-7 A, Figure 3-8 A and Table 3-3). There was no significant difference between the velocity and run length of KIF1A-S402A mutant in comparison to wild type KIF1A in tau $^{+/+}$ and tau $^{-/-}$ neurons (Figure 3-7 B, C and Figure 3-8 B, C). The non-phosphorylatable form of KIF1A-S402A was motile in the AβO-treated neurons which implies that S402 site might play an important part in regulation of KIF1A motility.

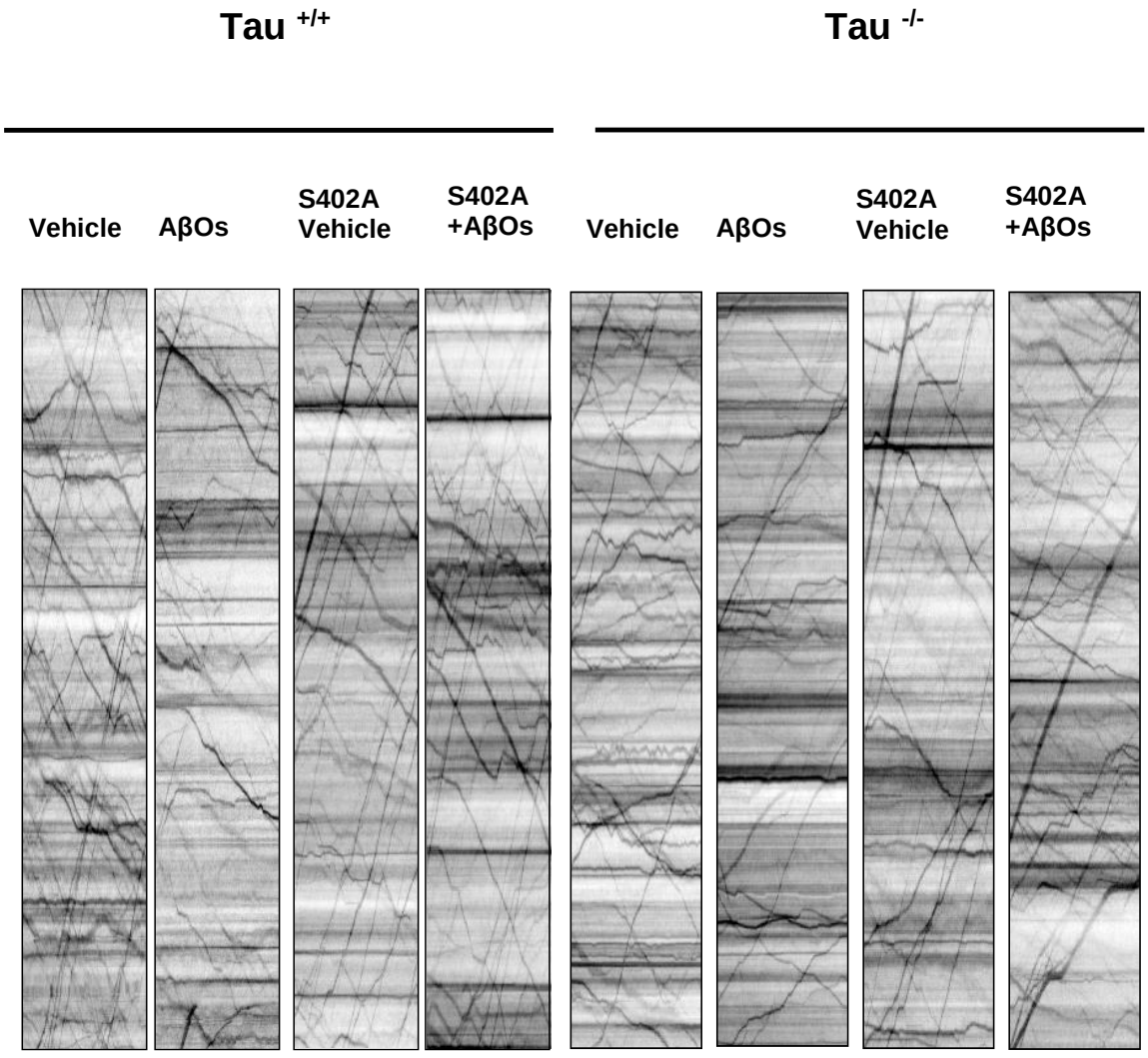
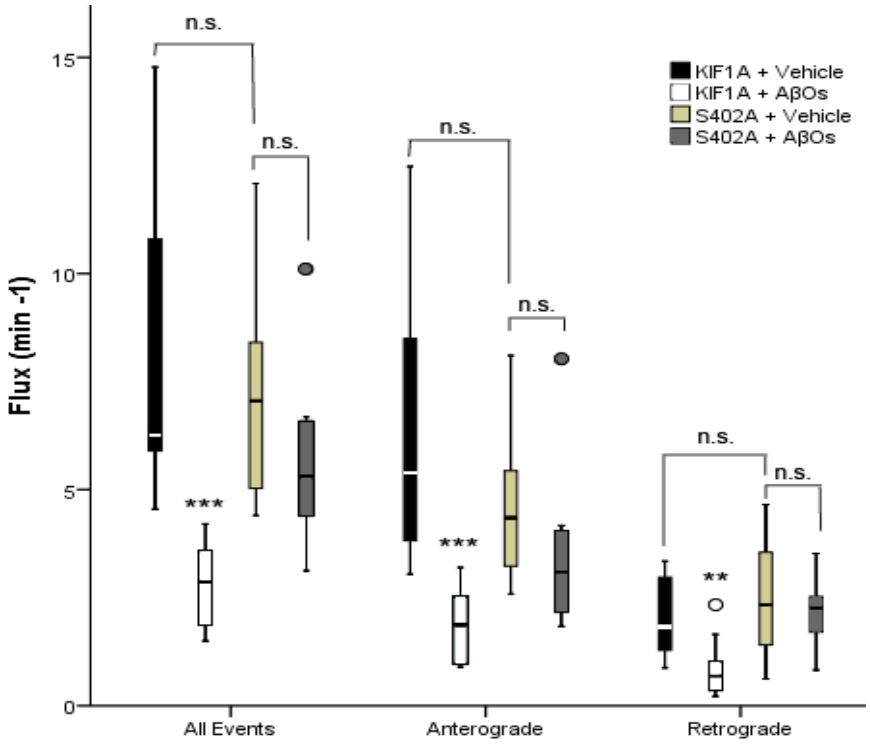


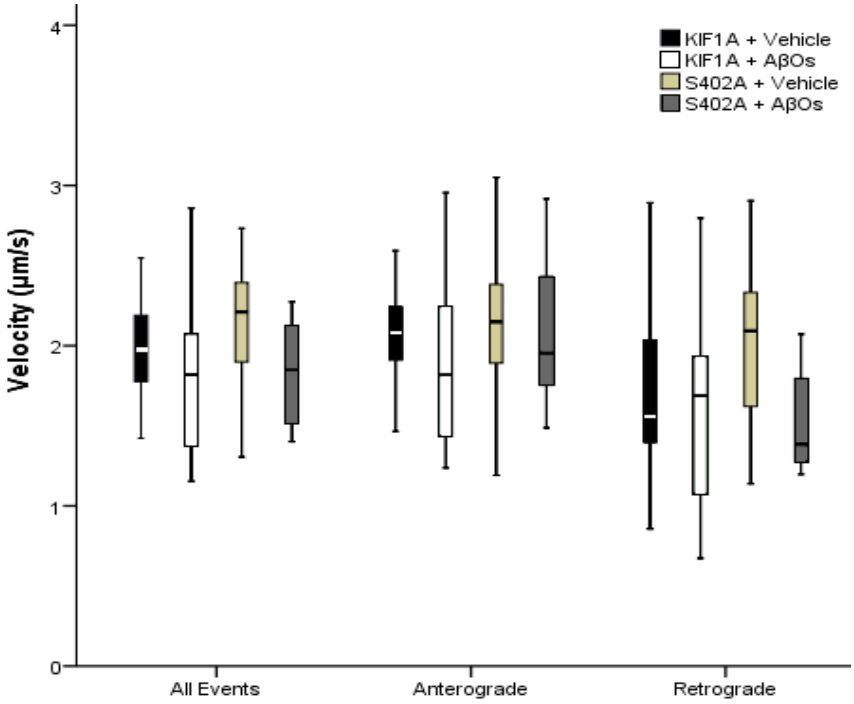
Figure 3-6: The phosphomutant KIF1A-S402A is motile in tau $^{+/+}$ and tau $^{-/-}$ AβO treated neurons.

Representative kymographs comparing KIF1A and KIF1A-S402A transport in vehicle and 500 nM AβO treated tau $^{+/+}$ and tau $^{-/-}$ neurons.

A) KIF1A-S402A Flux in tau $^{+/+}$ neurons



B) KIF1A-S402A Velocity



C) KIF1A-S402A Run Length

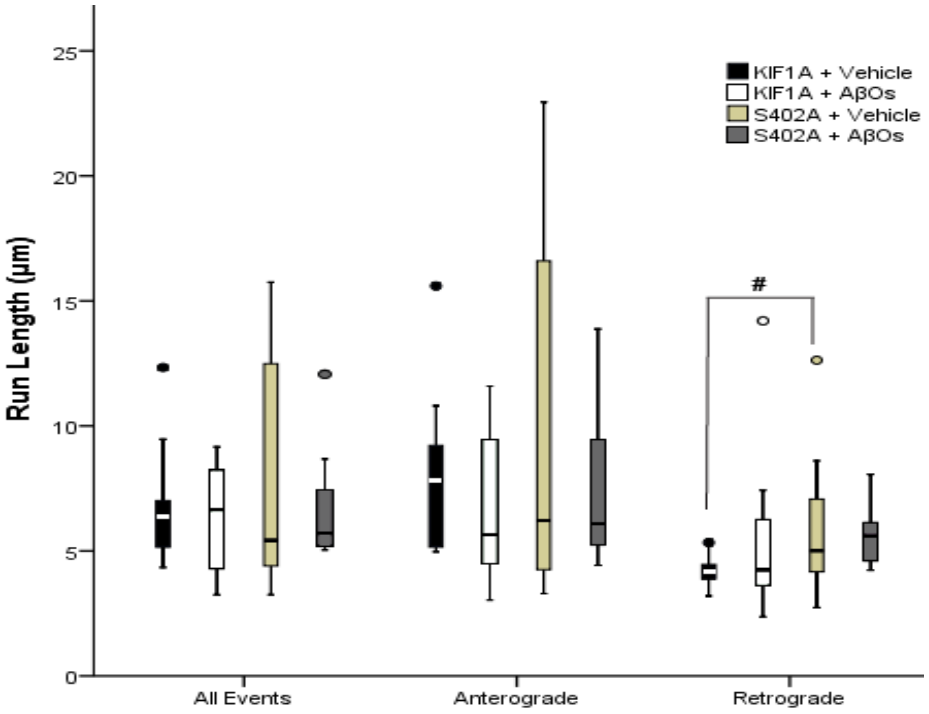
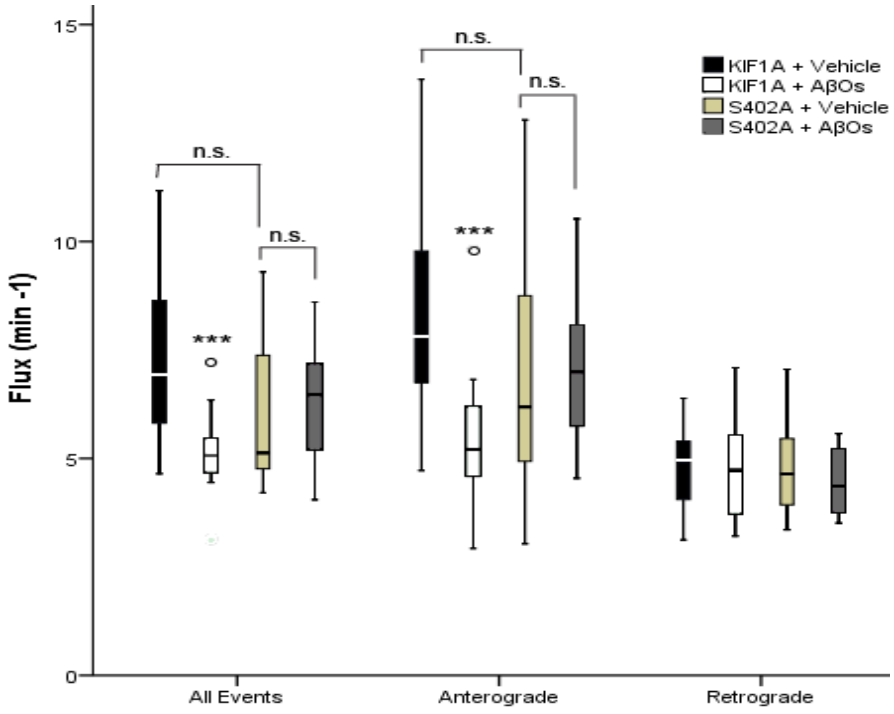


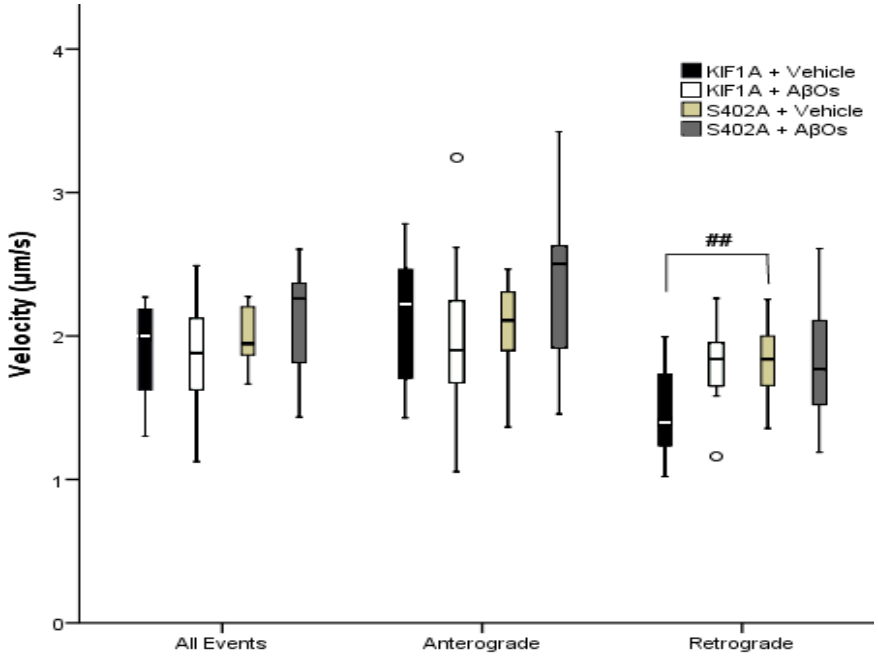
Figure 3-7: KIF1A and KIF1A-S402A transport dynamics comparison in tau $^{+/+}$ neurons treated with vehicle and AβOs

Effect of AβOs on KIF1A and KIF1A-S402A flux, velocity and run length in tau $^{+/+}$ neurons. Neurons were transfected with KIF1A-GFP and KIF1A-S402A-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβO treatment decreased the bidirectional flux of KIF1A in comparison to vehicle control. KIF1A-S402A transport was unaffected in AβO treated cells when compared to KIF1A-S402A vehicle treated cells. KIF1A and KIF1A-S402A transport is comparable B). The average velocity of KIF1A and KIF1A-S402A was unaffected by AβO treatment. C). AβO treatment had no effect on KIF1A and KIF1A-S402A run length. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared with S402A +vehicle

A) KIF1A-S402A Flux in tau $^{-/-}$ neurons



B) KIF1A-S402A Velocity



C) KIF1A-S402A Run length

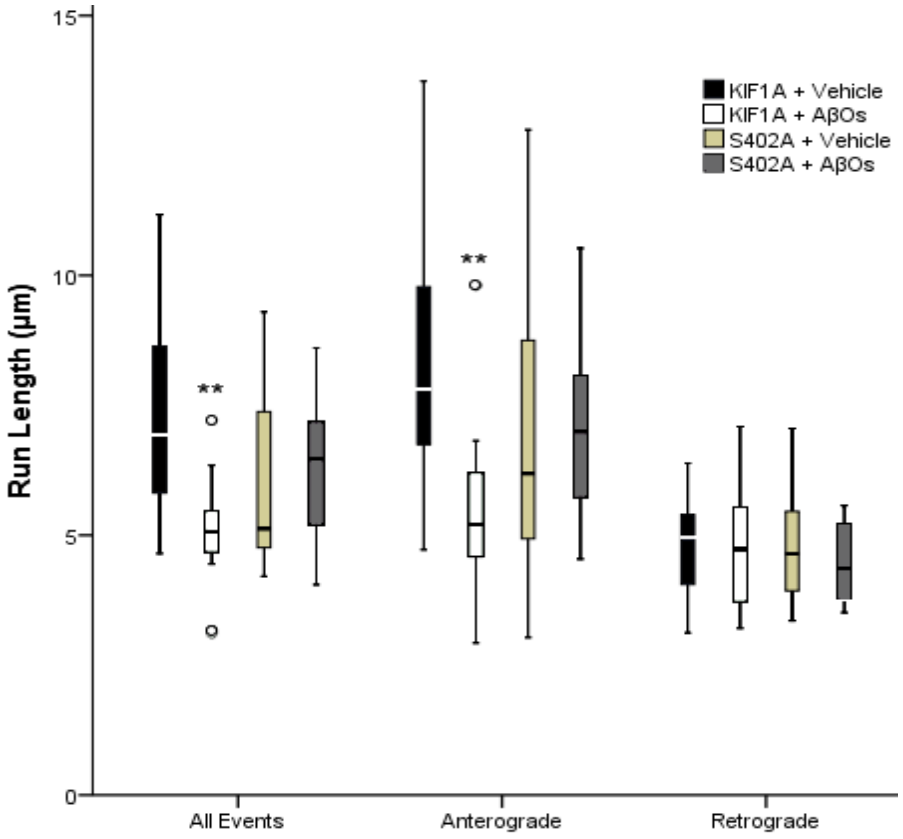


Figure 3-8: KIF1A and KIF1A-S402A transport dynamics comparison in tau $^{-/-}$ neurons treated with vehicle and AβOs

Effect of AβOs treatment on KIF1A and KIF1A-S402A flux, velocity and run length in tau $^{-/-}$ neurons. Neurons were transfected with KIF1A-GFP and KIF1A-S402A-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβO treatment decreased the anterograde flux of KIF1A in comparison to vehicle control. KIF1A-S402A transport was unaffected in AβO treated cells when compared to KIF1A-S402A vehicle treated cells. KIF1A and KIF1A-S402A transport is comparable B). The average velocity of KIF1A and KIF1A-S402A was unaffected by AβO treatment. But retrograde velocity of KIF1A is less than that of KIF1A-S402A C). AβO treatment had no effect on KIF1A-S402A run length but KIF1A run length in AβO treated cells is when compared to with vehicle treated cells. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared with S402A +vehicle.

Table 3-3: The phosphomutant KIF1A-S402A motility in AβOs treated neurons.

KIF1A axonal transport in hippocampal neurons				
	Traffic Values			%
	All Events	Anterograde	Retrograde	All Events
Flux (min^{-1})				
tau $^{+/+}$ KIF1A + vehicle	8.56 ± 0.94	6.48 ± 0.87	2.08 ± 0.24	100.00 ± 10.98
tau $^{+/+}$ KIF1A + AβOs	2.79 ± 0.28***	1.88 ± 0.23	0.90 ± 0.19**	32.59 ± 3.27***
tau $^{+/+}$ S402A +vehicle	7.15 ± 0.59	4.73 ± 0.46	2.43 ± 0.31	83.53 ± 6.89
tau $^{+/+}$ S402A + AβOs	5.72 ± 0.75	3.55 ± 0.70	2.16 ± 0.29	66.82 ± 8.76
tau $^{-/-}$ KIF1A + vehicle	8.58 ± 0.83	6.29 ± 0.72	2.29 ± 0.36	100.00 ± 9.67
tau $^{-/-}$ KIF1A + AβOs	3.70 ± 0.58 ***	1.97 ± 0.32***	1.73 ± 0.31	43.12 ± 6.75***
tau $^{-/-}$ S402A + vehicle	7.47 ± 0.64	4.73 ± 0.50	2.74 ± 0.36	87.06 ± 7.45
tau $^{-/-}$ S402A + AβOs	7.40 ± 0.87	5.01 ± 0.64	2.39 ± 0.43	86.25 ± 10.14
Velocity (µm/s)				
tau $^{+/+}$ KIF1A + vehicle	1.94 ± 0.08	2.06 ± 0.08	1.72 ± 0.14	100.00 ± 4.12
tau $^{+/+}$ KIF1A + AβOs	1.86 ± 0.15	1.96 ± 0.15	1.66 ± 0.17	95.87 ± 7.73
tau $^{+/+}$ S402A +vehicle	2.11 ± 0.10	2.14 ± 0.13	2.00 ± 0.13	108.76 ± 5.15
tau $^{+/+}$ S402A + AβOs	1.83 ± 0.12	2.08 ± 0.17	1.52 ± 0.12	94.32 ± 6.18
tau $^{-/-}$ KIF1A + vehicle	1.91 ± 0.08	2.11 ± 0.11	1.46 ± 0.08	100.00 ± 4.19
tau $^{-/-}$ KIF1A + AβOs	1.84 ± 0.10	1.92 ± 0.15	1.76 ± 0.08	96.33 ± 5.23
tau $^{-/-}$ S402A + vehicle	1.99 ± 0.06	2.08 ± 0.08	1.84 ± 0.06**	104.19 ± 3.14
tau $^{-/-}$ S402A + AβOs	2.11 ± 0.10	2.37 ± 0.15	1.83 ± 0.12	110.47 ± 5.23
Run Length (µm)				
tau $^{+/+}$ KIF1A + vehicle	6.58 ± 0.53	7.86 ± 0.74	4.23 ± 0.17	100.00 ± 8.05
tau $^{+/+}$ KIF1A + AβOs	6.36 ± 0.59	6.91 ± 0.80	5.41 ± 0.78	96.65 ± 8.96
tau $^{+/+}$ S402A +vehicle	8.22 ± 1.14	10.11 ± 1.71	5.79 ± 0.62	124.92 ± 17.32
tau $^{+/+}$ S402A + AβOs	6.72 ± 0.87	7.48 ± 1.20	5.62 ± 0.44	102.13 ± 13.22
tau $^{-/-}$ KIF1A + vehicle	7.20 ± 0.49	8.36 ± 0.62	4.81 ± 0.23	100.00 ± 6.80
tau $^{-/-}$ KIF1A + AβOs	5.07 ± 0.29**	5.43 ± 0.45**	4.68 ± 0.30	70.41 ± 4.03**
tau $^{-/-}$ S402A + vehicle	6.06 ± 0.45	7.10 ± 0.79	4.76 ± 0.28	84.17 ± 6.25
tau $^{-/-}$ S402A + AβOs	6.32 ± 0.42	7.10 ± 0.51	4.47 ± 0.22	87.78 ± 5.83

tau $^{+/+}$ KIF1A + vehicle: n = 15 kymographs (15 cells, 825 puncta), tau $^{+/+}$ KIF1A + AβOs: n = 14 kymographs (14 cells, 300 puncta), tau $^{+/+}$ S402A +vehicle: n = 16 kymographs (16 cells, 734 puncta), tau $^{+/+}$ S402A + AβOs: n = 8 kymographs (8 cells, 308 puncta), tau $^{-/-}$ KIF1A + vehicle: n = 15 kymographs (15 cells, 824 puncta), tau $^{-/-}$ KIF1A + AβOs: n = 14 kymographs (14 cells, 413 puncta), tau $^{-/-}$ S402A +vehicle: n = 14 kymographs (14 cells, 750 puncta), tau $^{-/-}$ S402A + AβOs: n = 12 kymographs (12 cells, 606 puncta)

*p<0.05, **p<0.01 and ***p<0.001 when compared with the KIF1A vehicle control.
$^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared with S402A +vehicle.

3.3.2. The phosphomimic KIF1A-S402E motility is reduced in AβO-treated neurons

To determine the potential role of KIF1A-S402 in transport regulation, a KIF1A-S402E-eGFP was expressed in mouse tau +/+ and tau -/- hippocampal neurons and compared to KIF1A-eGFP. The cells were treated with 500nM of AβOs 18 hrs prior to imaging. The representative kymographs show the difference between transport of KIF1A and KIF1A-S402E in control (vehicle treated) and AβO-treated neurons (Figure 3-9). The transport data showed unexpected results; we were expecting KIF1A-S402E mutant transport to be reduced instead it was unaffected and comparable to wild type KIF1A flux in vehicle treated neurons. In the AβO-treated neurons KIF1A-S402E transport was reduced by 66% ± 3.39 and 62% ± 5.09 in comparison to wild type KIF1A flux in tau $^{+/+}$ and tau $^{-/-}$ neurons, respectively (Figure 3-10 A, Figure 3-11 A and Table 3-4). A decrease in overall velocity of KIF1A-S402E mutant in comparison to wild type KIF1A in tau $^{+/+}$ and tau $^{-/-}$ neurons was also observed (Figure 3-10 B and Figure 3-11 B). In tau $^{-/-}$ AβO-treated neurons run length was decreased in comparison to vehicle treated KIF1A-S402E mutant while in tau $^{+/+}$ neurons run length was unaffected (Figure 3-10 C and Figure 3-11 C). These results implies that the KIF1A-S402E mutant might be interacting with endogenous wild type KIF1A, which may dimerize with KIF1A-S402E monomers and helping in its motility and hence, is masking the effect of the point mutation.

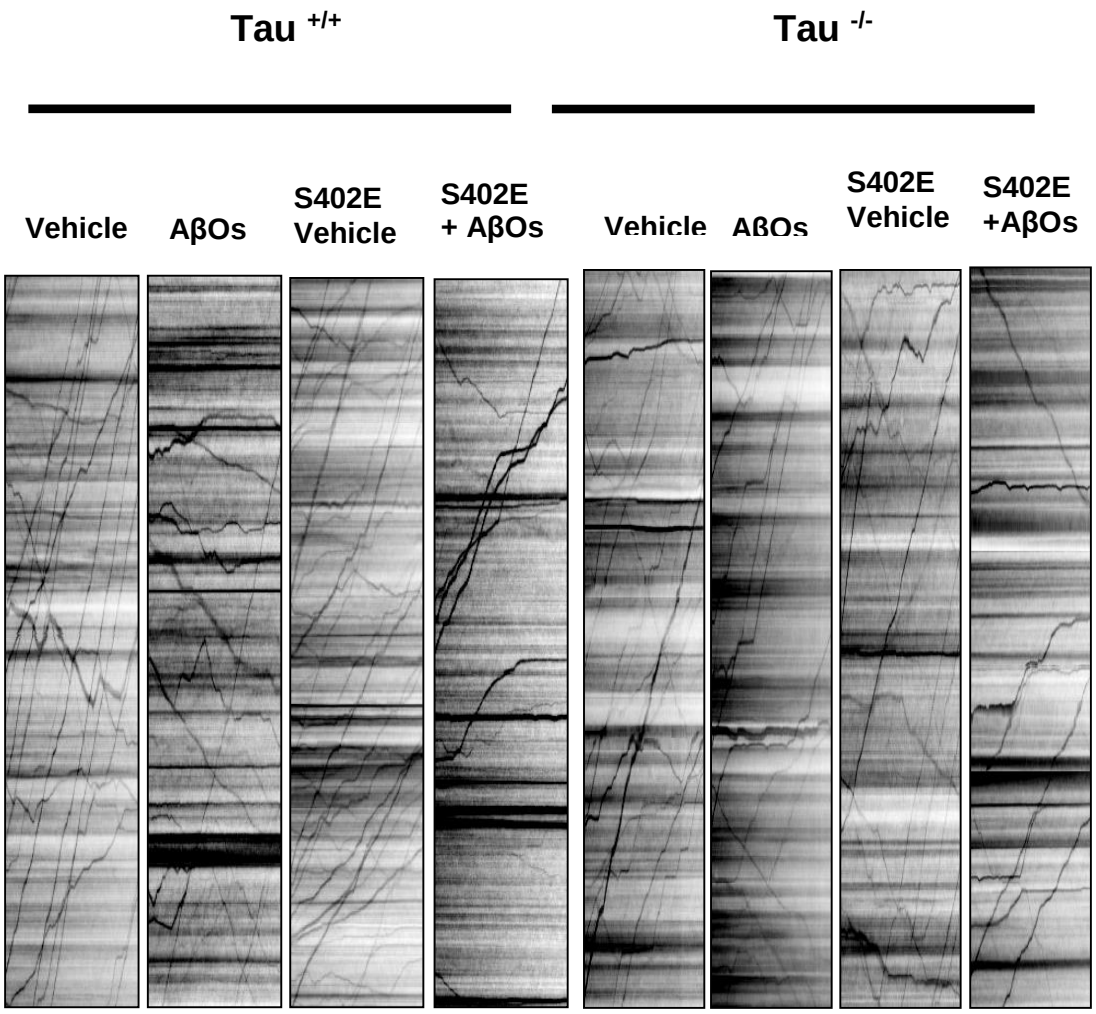
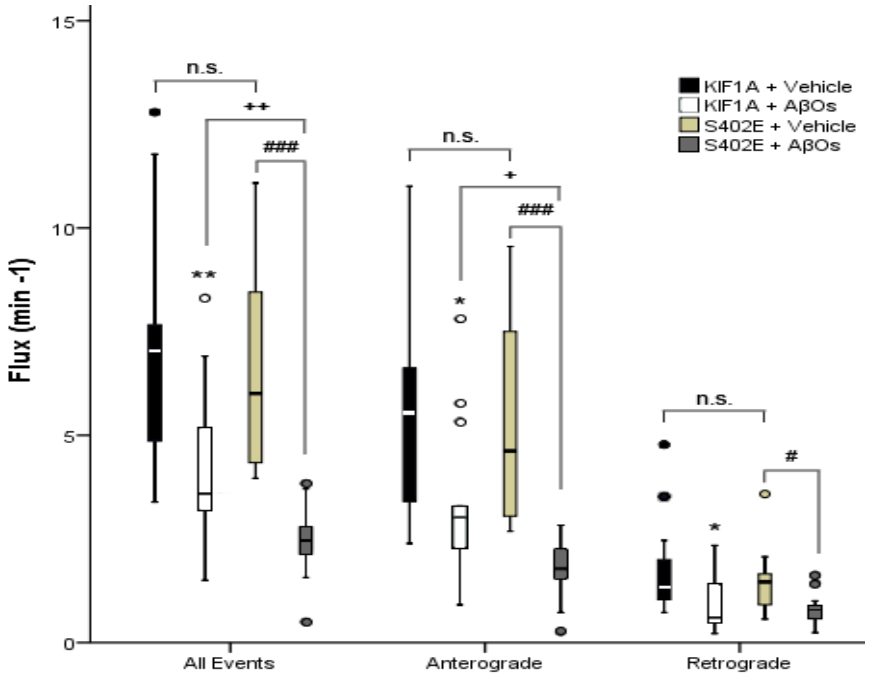


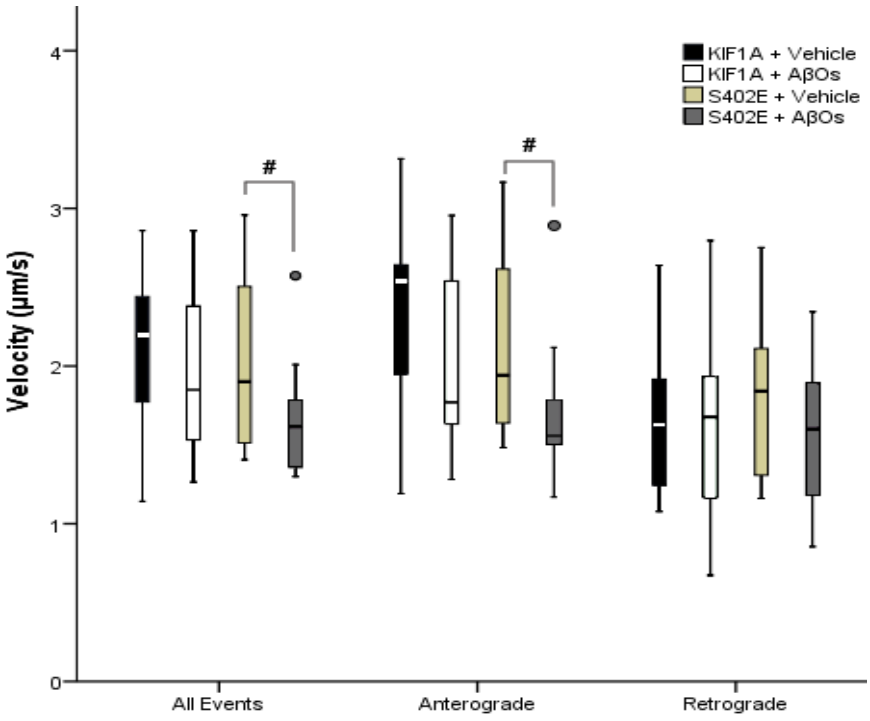
Figure 3-9: The phosphomimic KIF1A-S402E motility is severely reduced in AβO treated tau $^{+/+}$ and tau $^{-/-}$ neurons.

Representative kymographs comparing KIF1A and KIF1A-S402E transport in vehicle and 500 nM AβO treated tau $^{+/+}$ and tau $^{-/-}$ neurons.

A) KIF1A-S402E Flux in tau $^{+/+}$ neurons



B) KIF1A-S402E Velocity



C) KIF1A-S402E Run length

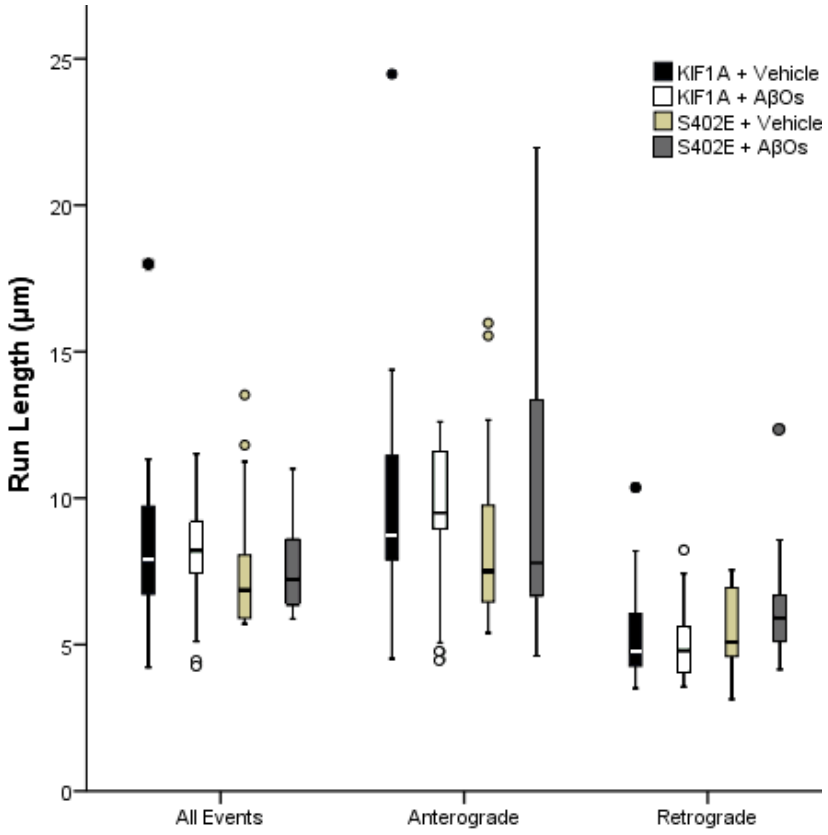
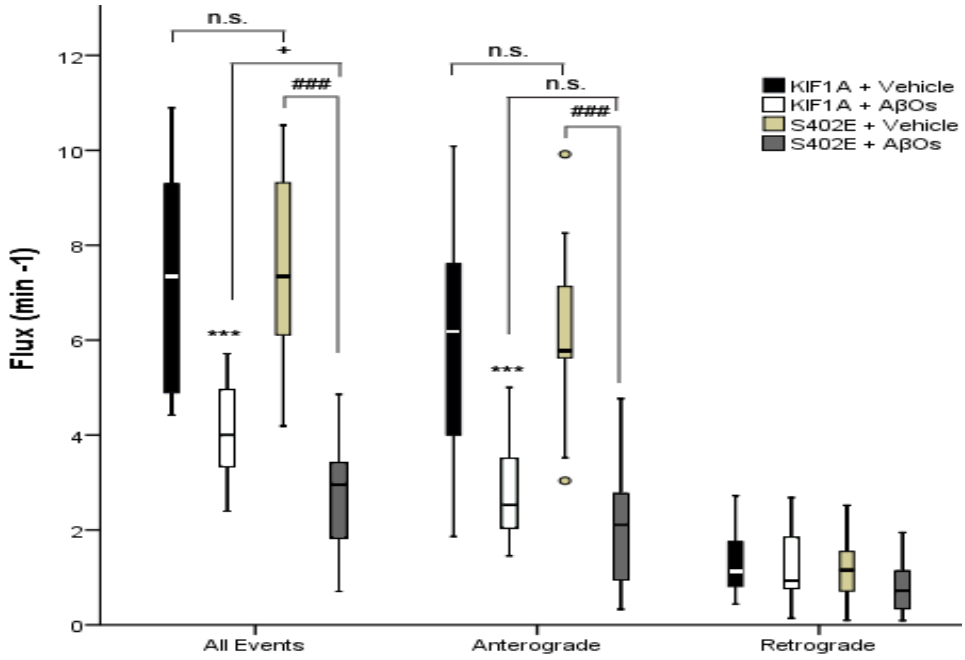


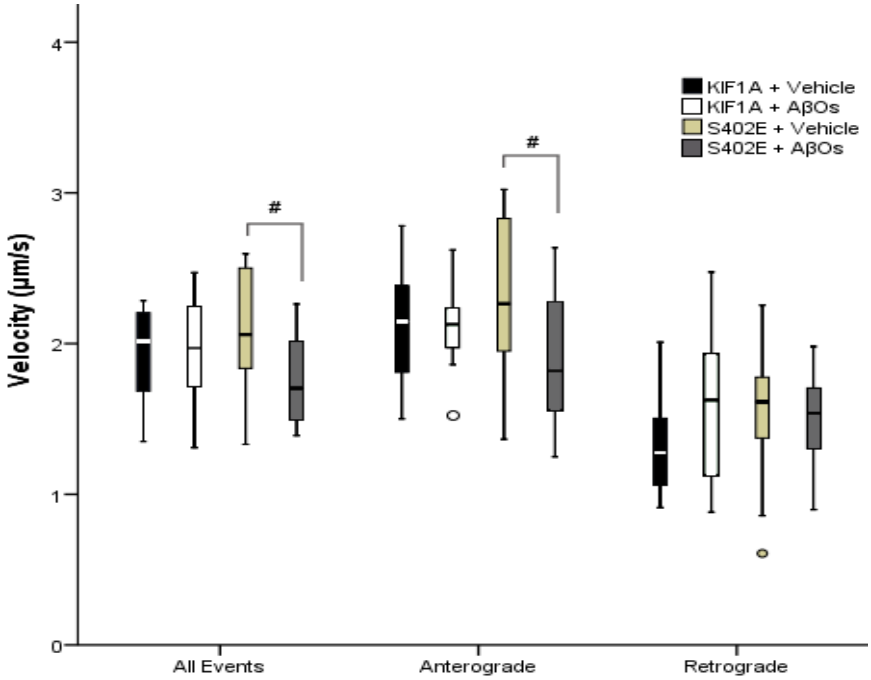
Figure 3-10: KIF1A and KIF1A-S402E transport dynamics comparison in tau $^{+/+}$ neurons treated with vehicle and AβOs

Effect of AβO treatment on KIF1A and KIF1A-S402E flux, velocity and run length in tau $^{+/+}$ neurons. Neurons were transfected with KIF1A-GFP and KIF1A-S402E-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβO treatment decreased the anterograde flux of KIF1A in comparison to vehicle control. KIF1A-S402E transport was reduced in AβO treated cells when compared to KIF1A-S402E vehicle control. KIF1A and KIF1A-S402E transport is comparable while in AβO treated cells KIF1A-S402E transport is severely reduced in comparison to KIF1A. B). The anterograde velocity of KIF1A was unaffected by AβOs treatment but KIF1A-S402E velocity is reduced in comparison to KIF1A-S402E control. But retrograde velocity of KIF1A is less than that of KIF1A-S402E C). AβO treatment had no effect on KIF1A and KIF1A-S402E run length. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared with S402E +vehicle. $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 when compared with KIF1A + AβOs.

A) KIF1A-S402E Flux tau $^{-/-}$ neurons



B) KIF1A-S402E Velocity



C) KIF1A-S402E Run length

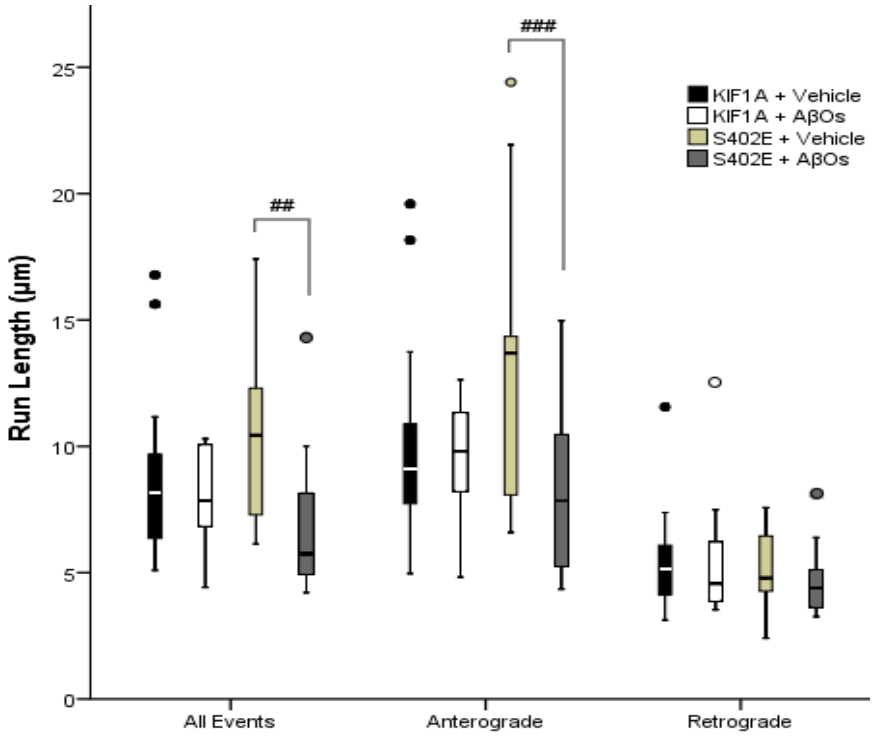


Figure 3-11: KIF1A and KIF1A-S402E transport dynamics comparison in tau $^{-/-}$ neurons treated with vehicle and AβOs

Effect of AβOs treatment on KIF1A and KIF1A-S402E flux, velocity and run length in tau $^{-/-}$ neurons. Neurons were transfected with KIF1A-GFP and KIF1A-S402E-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. A). AβO treatment decreased the anterograde flux of KIF1A in comparison to vehicle control. KIF1A-S402E transport was severely reduced in AβO treated cells when compared to KIF1A-S402E vehicle control. KIF1A and KIF1A-S402E transport is comparable while in AβOs treated cells KIF1A-S402E transport is severely reduced in comparison to KIF1A. B).The average velocity of KIF1A was unaffected by AβO treatment but KIF1A-S402E velocity is reduced in comparison to KIF1A-S402E control. C). AβO treatment reduced KIF1A-S402E run length as compared to KIF1A-S402E control while it had no effect on KIF1A run length. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the vehicle control. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared with S402E +vehicle. $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 when compared with KIF1A + AβOs.

Table 3-4: The phosphomimic KIF1A-S402E motility in AβOs treated neurons.

KIF1A axonal transport in hippocampal neurons

	Traffic Values			%
	All Events	**Anterograde**	**Retrograde**	**All Events**
Flux (min^{-1})				
tau $^{+/+}$ KIF1A + vehicle	7.08 ± 0.76	5.31 ± 0.61	1.76 ± 0.28	100.00 ± 10.73
tau $^{+/+}$ KIF1A + AβOs	4.26 ± 0.55**	3.32 ± 0.53*	0.94 ± 0.19*	60.17 ± 7.77**
tau $^{+/+}$ S402E +vehicle	6.73 ± 0.72	5.23 ± 0.67	1.49 ± 0.22	95.06 ± 10.16
tau $^{+/+}$ S402E + AβOs	2.43 ± 0.24$^{###}$	1.81 ± 0.20$^{###}$	0.79 ± 0.11$^{#}$	34.32 ± 3.39$^{###}$
tau $^{-/-}$ KIF1A + vehicle	7.27 ± 0.63	6.00 ± 0.63	1.27 ± 0.19	100.00 ± 8.66
tau $^{-/-}$ KIF1A + AβOs	4.03 ± 0.30***	2.80 ± 0.29***	1.22 ± 0.23	55.43 ± 4.12***
tau $^{-/-}$ S402E + vehicle	7.41 ± 0.52	6.19 ± 0.52	1.22 ± 0.19	101.92 ± 7.15
tau $^{-/-}$ S402E + AβOs	2.82 ± 0.37$^{###}$	2.01 ± 0.36$^{###}$	0.81 ± 0.17	38.79 ± 5.09$^{###}$
Velocity (µm/s)				
tau $^{+/+}$ KIF1A + vehicle	2.09 ± 0.13	2.31 ± 0.16	1.68 ± 0.12	100.00 ± 6.22
tau $^{+/+}$ KIF1A + AβOs	1.89 ± 0.14	2.01 ± 0.15	1.62 ± 0.16	90.43 ± 6.70
tau $^{+/+}$ S402E +vehicle	2.06 ± 0.15	2.17 ± 0.17	1.82 ± 0.14	98.56 ± 7.17
tau $^{+/+}$ S402E + AβOs	1.67 ± 0.10$^{#}$	1.71 ± 0.12$^{#}$	1.56 ± 0.14	79.90 ± 4.78$^{#}$
tau $^{-/-}$ KIF1A + vehicle	1.92 ± 0.08	2.14 ± 0.11	1.33 ± 0.08	100.00 ± 4.17
tau $^{-/-}$ KIF1A + AβOs	1.94 ± 0.10	2.12 ± 0.09	1.59 ± 0.14	101.04 ± 5.20
tau $^{-/-}$ S402E + vehicle	2.08 ± 0.11	2.32 ± 0.15	1.52 ± 0.13	108.3 ± 5.73
tau $^{-/-}$ S402E + AβOs	1.77 ± 0.09$^{#}$	1.88 ± 0.13$^{#}$	1.50 ± 0.09	92.18 ± 4.68
Run Length (µm)				
tau $^{+/+}$ KIF1A + vehicle	8.43 ± 0.85	10.23 ± 1.22	5.43 ± 0.49	100.00 ± 10.08
tau $^{+/+}$ KIF1A + AβOs	7.96 ± 0.60	9.33 ± 0.79	5.03 ± 0.39	94.42 ± 7.12
tau $^{+/+}$ S402E +vehicle	7.93 ± 0.72	9.06 ± 0.99	5.56 ± 0.42	94.06 ± 8.54
tau $^{+/+}$ S402E + AβOs	7.63 ± 0.43	10.63 ± 1.53	6.33 ± 0.59	90.51 ± 5.10
tau $^{-/-}$ KIF1A + vehicle	8.93 ± 0.94	10.15 ± 1.13	5.44 ± 0.56	100.00 ± 10.52
tau $^{-/-}$ KIF1A + AβOs	7.99 ± 0.56	9.50 ± 0.69	5.45 ± 0.74	89.47 ± 6.27
tau $^{-/-}$ S402E + vehicle	10.69 ± 1.05	13.49 ± 1.57	5.12 ± 0.41	119.70 ± 11.75
tau $^{-/-}$ S402E + AβOs	6.84 ± 0.86$^{##}$	8.11 ± 0.92$^{###}$	4.69 ± 0.40	76.59 ± 9.63$^{##}$

tau $^{+/+}$ KIF1A + vehicle: n = 15 kymographs (15 cells, 659 puncta), tau $^{+/+}$ KIF1A + AβOs: n = 13 kymographs (13 cells, 346 puncta), tau $^{+/+}$ S402E +vehicle: n = 13 kymographs (13 cells, 559 puncta), tau $^{+/+}$ S402E + AβOs: n = 13 kymographs (13 cells, 198 puncta), tau $^{-/-}$ KIF1A + vehicle: n = 14 kymographs (14 cells, 604 puncta), tau $^{-/-}$ KIF1A + AβOs: n = 12 kymographs (12 cells, 312 puncta), tau $^{-/-}$ S402E +vehicle: n = 13 kymographs (13 cells, 478 puncta), tau $^{-/-}$ S402E + AβOs: n = 12 kymographs (12 cells, 253 puncta)

*p<0.05, **p<0.01 and ***p<0.001 when compared with the KIF1A vehicle control.

$^{#}$p<0.05, $^{##}$p<0.01 and $^{###}$p<0.001 when compared with S402E +vehicle.

3.3.2.1 KIF1A-S402E transport rescued by GSK3β inhibition

Unpublished data from our lab shows that GSK3β inhibitor VIII rescues the transport of wild type KIF1A in AβO-treated neurons. GSK3β Inhibitor VIII is a cell-permeable, potent, and selective inhibitor of GSK3β. To determine the potential role of GSK3β inhibitor VIII in rescuing the transport of KIF1A-S402E mutant, a KIF1A-S402E-eGFP was expressed in rat hippocampal neurons. The cells were treated with 500nM of AβOs 18 hrs prior to imaging. GSK3β inhibitor VIII was applied 30 minutes prior to imaging the cells. The representative kymographs show the differences between transport of KIF1A-S402E in control (vehicle treated), AβO-treated and GSK3β inhibitor VIII and AβO-treated neurons (Figure 3-12). The AβO treatment reduced the flux of KIF1A-S402E mutant by 68%, but the GSK3β inhibitor VIII rescued 80% ± 7.75 of the transport defects and its flux was comparable to control KIF1A-S402E (vehicle treated) (Figure 3-13 A and Table 3-5). The velocity and run length of GSK3β inhibitor VIII and AβO-treated neurons was unaffected in comparison to KIF1A-S402E (vehicle treated) control (Figure 3-13 B and C). Hence, KIF1A-S402 is potential site of GSK3β phosphorylation and KIF1A-S402E transport rescue by the GSK3β inhibitor shows that this site is possibly playing a role in modulating KIF1A motility. Though data show that GSK3β inhibitor VIII is rescuing transport, it is not clear whether GSK3β inhibitor VIII is acting on endogenous KIF1A or the KIF1A-S402E mutant.

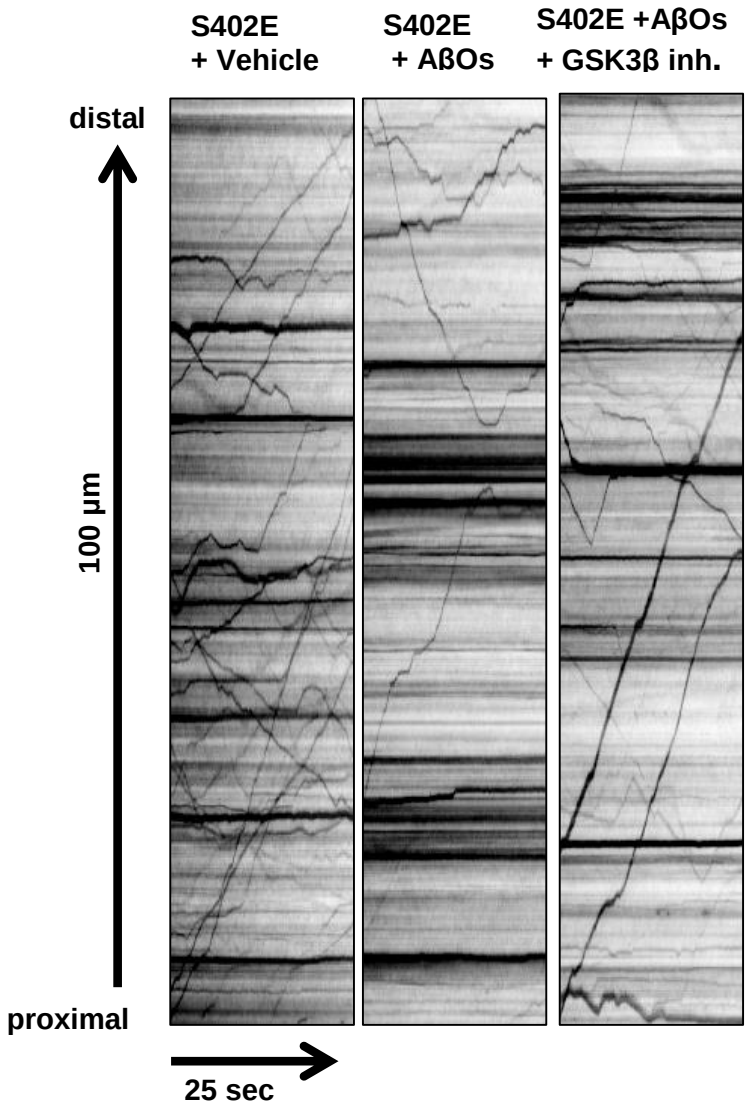
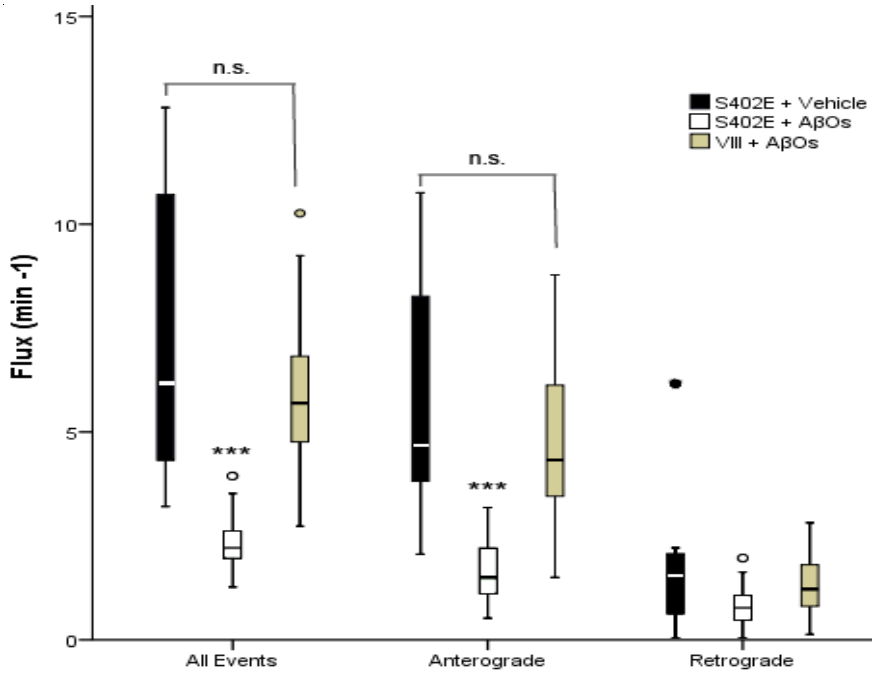


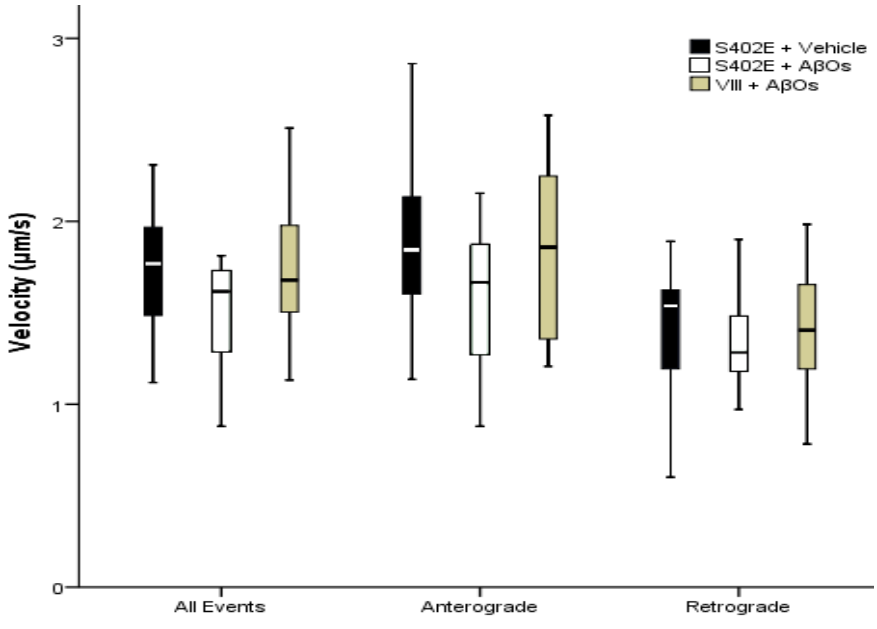
Figure 3-12: GSK3β inhibitor VIII rescues the transport defects introduced by AβOs.

Representative kymographs comparing KIF1A-S402E transport in vehicle, 500 nm AβOs and 5 uM inhibitor treated neurons.

A) KIF1A-S402E Flux in the presence of GSK3β inhibitor VIII + AβOs



B) KIF1A-S402E Velocity



C) KIF1A-S402E Run length

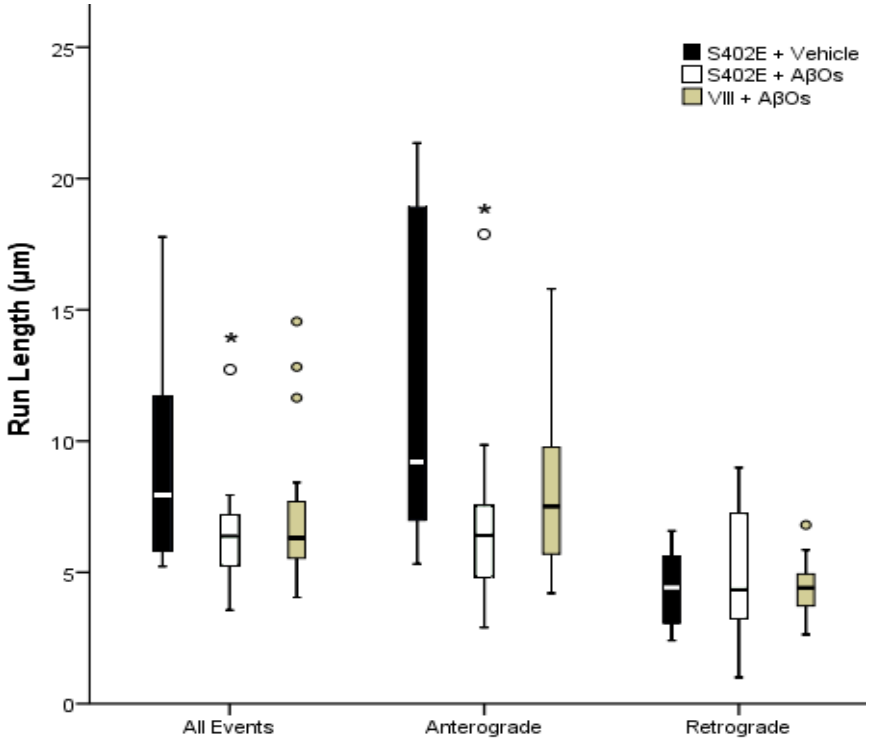


Figure 3-13: KIF1A-S402E transport dynamics in AβOs and GSK3β inhibitor VIII treated neurons.

Effect of AβOs and GSK3β inhibitor VIII treatment on KIF1A-S402E flux, velocity and run length in hippocampal neurons. Neurons were transfected with KIF1A-S402E-GFP for 24 hours and then treated with 500 nM AβOs (or with vehicle) for 18 hrs. GSK3β inhibitor VIII was applied 30 min before imaging. A). AβO treatment decreased the average and anterograde flux of KIF1A-S402E in comparison to KIF1A-S402E vehicle control while GSK3β inhibitor VIII treatment rescued the KIF1A-S402E transport. B). The average velocity of KIF1A was unaffected by AβOs treatment and GSK3β inhibitor VIII treatment. C). AβO treatment reduced KIF1A-S402E run length as compared to KIF1A-S402E control. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the KIF1A-S402E vehicle control.

Table 3-5: The phosphomimic KIF1A-S402E motility in AβOs and GSK3β inhibitor VIII treated neurons.

KIF1A axonal transport in hippocampal neurons

	Traffic Values			%
	All Events	**Anterograde**	**Retrograde**	**All Events**
Flux (min^{-1})				
S402E +vehicle	7.35 ± 0.84	5.78 ± 0.75	1.57 ± 0.38	100.00 ± 11.42
S402E + AβOs	2.37 ± 0.17***	1.65 ± 0.20***	0.73 ± 0.15	32.24 ± 2.31***
VIII + AβOs	5.88 ± 0.57	4.58 ± 0.57	1.29 ± 0.19	80.00 ± 7.75
Velocity (µm/s)				
S402E +vehicle	1.71 ± 0.09	1.87 ± 0.12	1.40 ± 0.08	100.00 ± 5.26
S402E + AβOs	1.49 ± 0.08	1.56 ± 0.10	1.21 ± 0.14	87.13 ± 4.67
VIII + AβOs	1.74 ± 0.10	1.83 ± 0.12	1.46 ± 0.13	101.75 ± 5.84
Run Length (µm)				
S402E +vehicle	9.19 ± 1.08	11.87 ± 1.61	4.50 ± 0.37	100.00 ± 11.75
S402E + AβOs	6.45 ± 0.58	6.82 ± 0.91	4.70 ± 0.73	70.18 ± 6.31
VIII + AβOs	7.35 ± 0.82*	8.38 ± 0.93*	4.47 ± 0.28	79.97 ± 8.92*

S402E + vehicle: n = 15 kymographs (15 cells, 675 puncta)
S402E + AβOs: n = 15 kymographs (15 cells, 298 puncta)
VIII + AβOs: n = 15 kymographs (15 cells, 643 puncta)

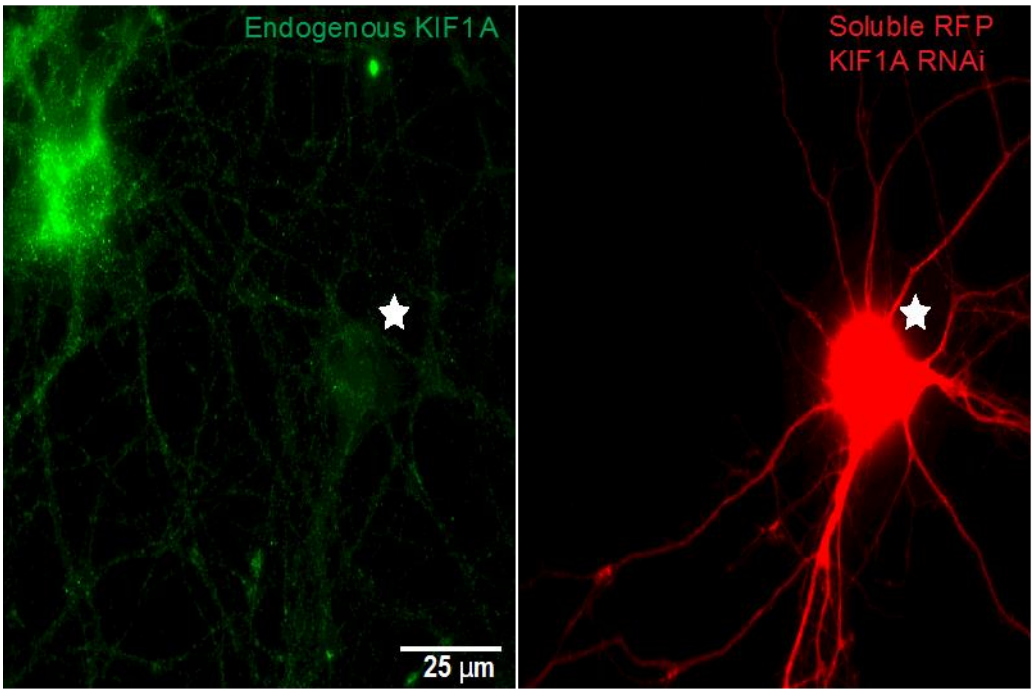
*p<0.05 when compared with the S402E vehicle control.
**p<0.01 when compared with the S402E vehicle control.
***p<0.001 when compared with the S402E vehicle control.

3.4. KIF1A expression is reduced in the KIF1A knockdown neurons

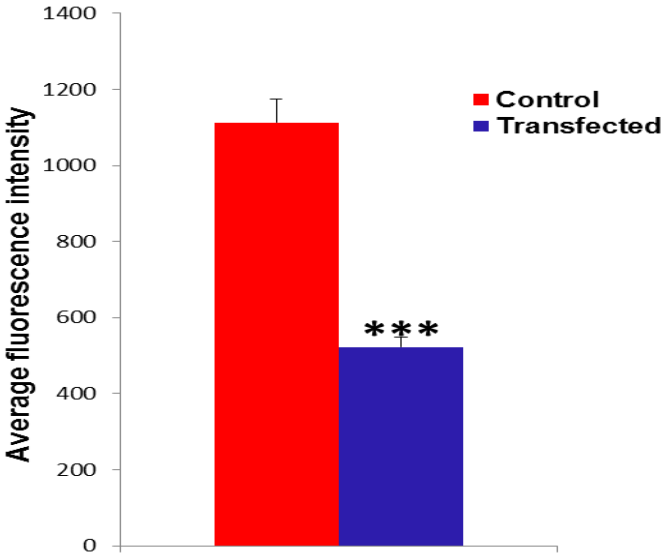
To test if endogenous wild type KIF1A influences KIF1A-S402E motility I used RNAi technology to knockdown endogenous KIF1A. I conducted two different set of experiments in endogenous wild type KIF1A knocked down hippocampal neurons. First, I performed quantitative immunocytochemistry to determine the extent of endogenous KIF1A reduction in RNAi treated cells, while in the other experiment I asked if KIF1A-S402E transport dynamics are altered in KIF1A knockdown cells in the absence of AβOs.

To determine KIF1A expression levels after RNAi treatment, I performed immunocytochemistry. I expressed KIF1A-RNAi-RFP in the hippocampal neurons and the cells showed reduced levels of endogenous KIF1A as compared to untransfected cells (Figure 3-14 A and B). This data shows that the endogenous KIF1A expression levels are reduced by approximately 53 % when KIF1A-RNAi is expressed in the neurons.

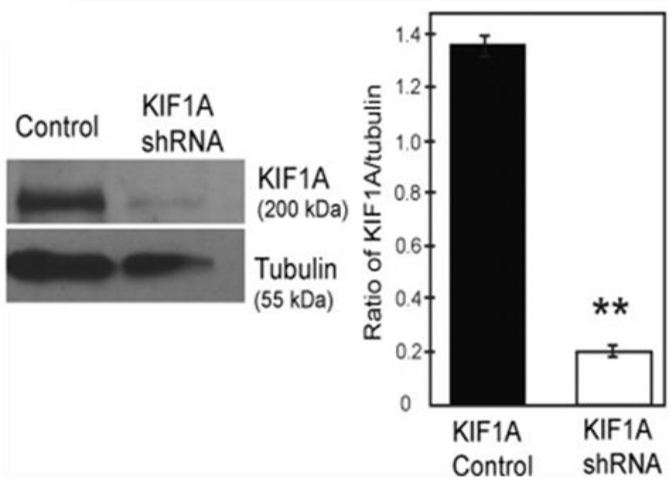
A.



B.



C.



Lo et al., 2011

Figure 3-14: Endogenous KIF1A expression is reduced in RNAi transfected neurons.

A). Immunocytochemistry shows that the expression of endogenous KIF1A (green) is reduced in KIF1A RNAi transfected (red) neurons (star denotes transfected cell). B). Quantification of the KIF1A expression in untransfected (control) versus endogenous KIF1A knockdown neurons. C). Our lab has previously shown by immunoblotting that transfecting neurons with KIF1A-RNAi results in reduction of KIF1A expression levels in comparison to untransfected neurons. Quantification of the knockdown of KIF1A is expressed as a ratio of KIF1A to tubulin expression (Lo et al., 2011).

To determine if endogenous wild type KIF1A influences KIF1A-S402E motility I transfected the cells with a plasmid expressing RNAi against wild type KIF1A along with an RNAi-resistant form of KIF1A-S402E. The representative kymographs show the transport pattern of KIF1A-S402E in RNAi expressing neurons in comparison to KIF1A wild type (vehicle treated) control (Figure 3-15). In comparison to wild type KIF1A, the flux of KIF1A-S402E is reduced by 53% ± 6.72 in the absence of endogenous KIF1A (Figure 3-16 A and Table 3-6) while the velocity and run length of KIF1A-S402E in endogenous KIF1A knock down neurons was unaffected and comparable to wild type KIF1A (Figure 3-16 B and C). Hence, the phosphomimic KIF1A- S402E transport is significantly reduced even in the absence of AβOs. Taken together, the KIF1A transport data imply that KIF1A-S402 is a critical residue in the regulation of KIF1A (Table 3-7).

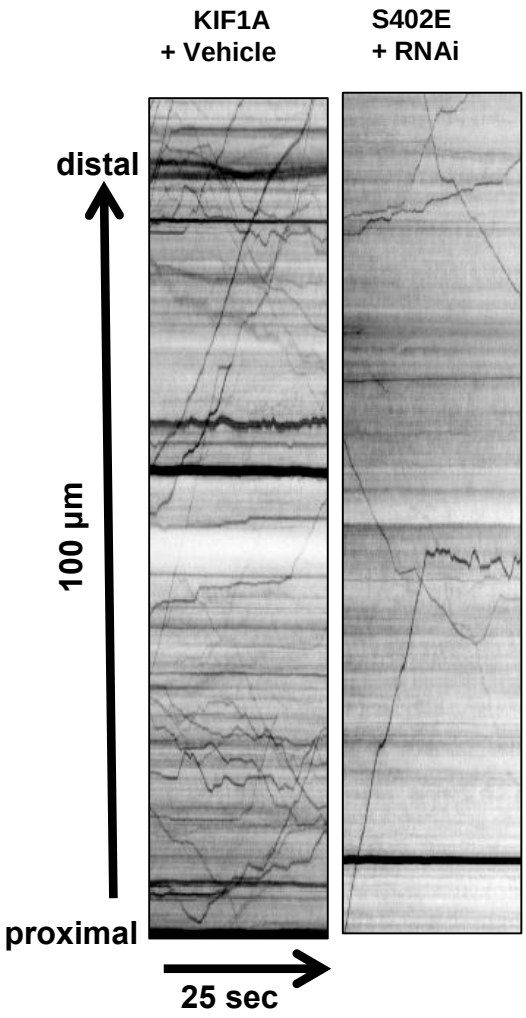
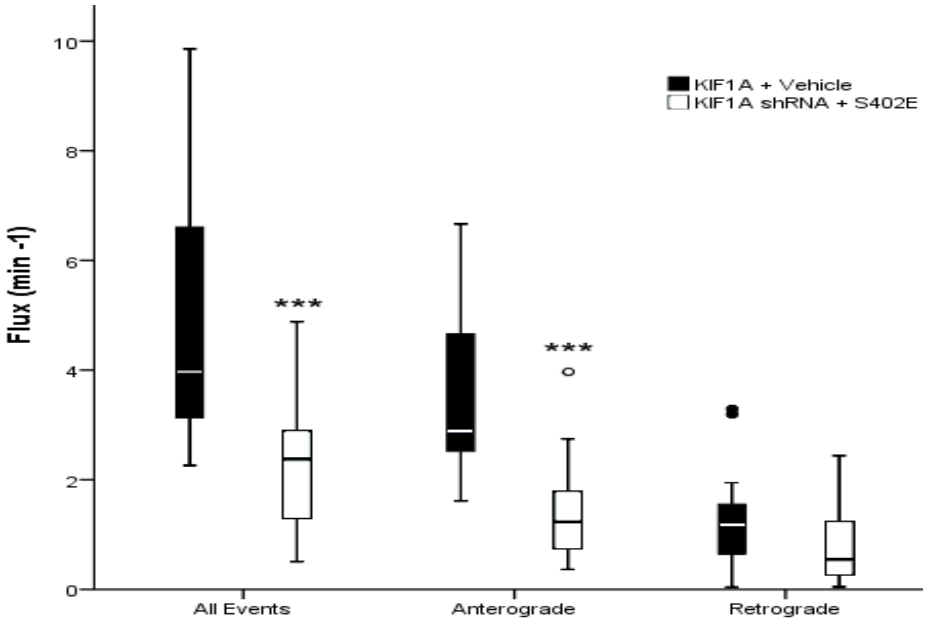


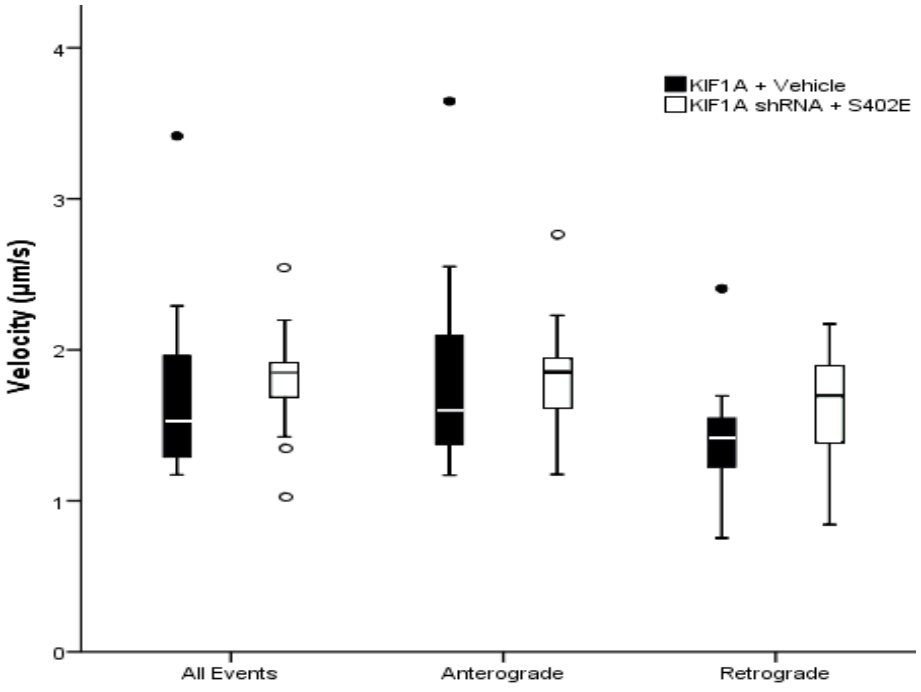
Figure 3-15: KIF1A-S402E transport is reduced in KIF1A siRNA expressing neurons.

A). Representative kymographs comparing wild type KIF1A transport in vehicle treated neurons with KIF1A-S402E transport in endogenous KIF1A knock down neurons. Neuronal cells were transfected with KIF1A and KIF1A-S402E + KIF1A shRNAi plasmid for 24 hours.

A) KIF1A-S402E Flux in KIF1A shRNA treated neurons



B) KIF1A-S402E Velocity



C) KIF1A-S402E Run length

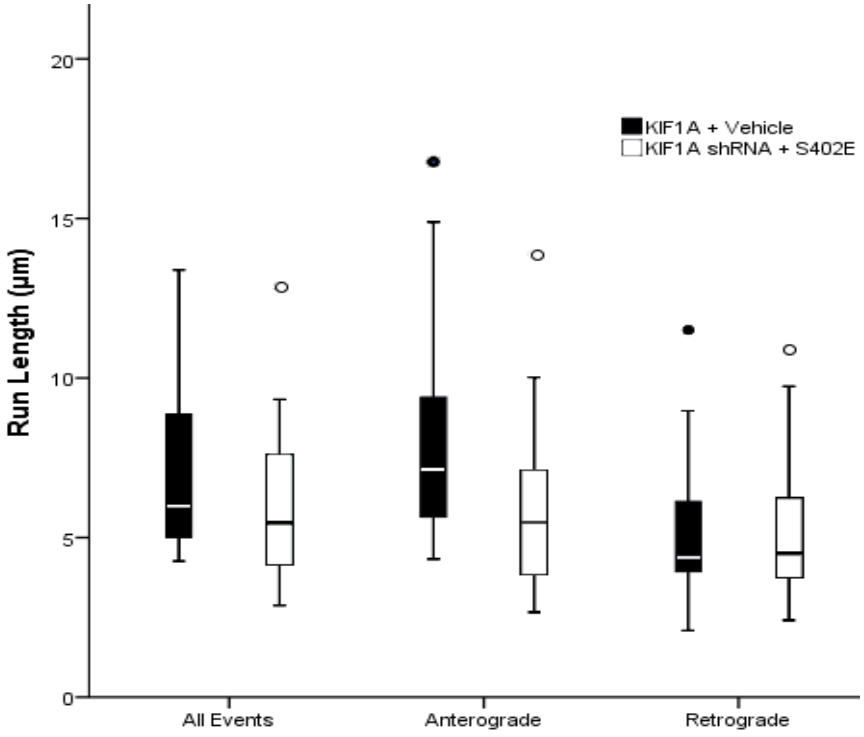


Figure 3-16: KIF1A-S402E transport dynamics in KIF1A knockdown neurons.
Effect of knocking down endogenous KIF1A on KIF1A-S402E flux, velocity and run length in hippocampal neurons. Neurons were transfected with KIF1A-GFP and KIF1A-S402E-GFP + KIF1A shRNA for 24 hours and then imaged. A). Knocking down endogenous KIF1A caused a reduction in anterograde flux of KIF1A-S402E in comparison to KIF1A vehicle control. B). The endogenous KIF1A knockdown had no effect on average velocity of KIF1A-S402E in comparison to KIF1A vehicle control. C). The endogenous KIF1A knock down had no effect on average run length of KIF1A-S402E in comparison to KIF1A vehicle control. A minimum of 12 cells per condition from three different cultures were analyzed. *p<0.05, **p<0.01 and ***p<0.001 when compared with the KIF1A vehicle control.

Table 3-6: The phosphomimic KIF1A-S402E motility in KIF1A knockdown neurons.

KIF1A axonal transport in hippocampal neurons				
	Traffic Values			%
	All Events	Anterograde	Retrograde	All Events
Flux (min^{-1})				
KIF1A + vehicle	4.76 ± 0.51	3.55 ± 0.37	1.21 ± 0.21	100.00 ± 10.71
shRNA + S402E	2.25 ± 0.32***	1.45 ± 0.26***	0.79 ± 0.18	47.26 ± 6.72***
Velocity (µm/s)				
KIF1A + vehicle	1.69 ± 0.12	1.81 ± 0.14	1.40 ± 0.08	100.00 ± 7.100
shRNA + S402E	1.79 ± 0.09	1.82 ± 0.01	1.62 ± 0.10	105.91 ± 5.32
Run Length (µm)				
KIF1A + vehicle	7.14 ± 0.65	8.10 ± 0.81	4.99 ± 0.53	100.00 ± 9.10
shRNA + S402E	6.07 ± 0.71	6.12 ± 0.79	5.41 ± 0.64	85.01 ± 9.94

KIF1A + vehicle: n = 19 kymographs (19 cells, 620 puncta)
shRNA + S402E: n = 15 kymographs (15 cells, 289 puncta)

*p<0.05 when compared with the KIF1A vehicle control.
**p<0.01 when compared with the KIF1A vehicle control.
***p<0.001 when compared with the KIF1A vehicle control.

Table 3-7: KIF1A axonal transport summary

KIF1A axonal transport in hippocampal neurons

Motor genotypes	Tau $^{+/+}$	Tau $^{-/-}$	AβOs	% Flux (min^{-1})	Transport relative to control`
KIF1A	+	-	-	100.00 ± 10.73	
KIF1A	+	-	+	60.17 ± 7.77**	↓
KIF1A	-	+	-	100.00 ± 8.66	=
KIF1A	-	+	+	55.43 ± 4.12***	↓
KIF1A- S402 A	+	-	-	83.53 ± 6.89	=
KIF1A- S402 A	+	-	+	66.82 ± 8.76	≈
KIF1A- S402 A	-	+	-	87.06 ± 7.45	=
KIF1A- S402 A	-	+	+	86.25 ± 10.14	≈
KIF1A- S402 E	+	-	-	95.06 ± 10.16	=
KIF1A- S402 E	+	-	+	34.32 ± 3.39$^{###, ++}$	↓↓
KIF1A- S402 E	-	+	-	101.92 ± 7.15	=
KIF1A- S402 E	-	+	+	38.79 ± 5.09$^{###, +}$	↓↓
KIF1A- S402 E^{VIII}	+	-	-	80.00 ± 7.75	≈
KIF1A-S402E^{RNAi}	+	-	-	47.26 ± 6.72***	↓

*p<0.05, **p<0.01 and ***p<0.001 when compared with the KIF1A without AβOs.
#p<0.05, ##p<0.01 and ###p<0.001 when compared with S402E +vehicle.
+p<0.05, ++p<0.01 and +++p<0.001 when compared with KIF1A + AβOs

↓	reduced
=	equal
≈	Approximately equal
↓↓	markedly reduced

3.5. KIF1A binds GSK3β in cultured neurons

Experiments from our lab using GSK3β inhibitors indicate that KIF1A is a potential substrate for GSK3β (Ramser and Gan et al., 2013). In order to discover if there was a physical interaction between KIF1A and GSK3β, KIF1A was immunoprecipitated from the cell lysates of mouse hippocampal neurons, and immunoblotting with an anti-GSK3β antibody suggests that these two proteins do interact with each other (Figure 3-17).

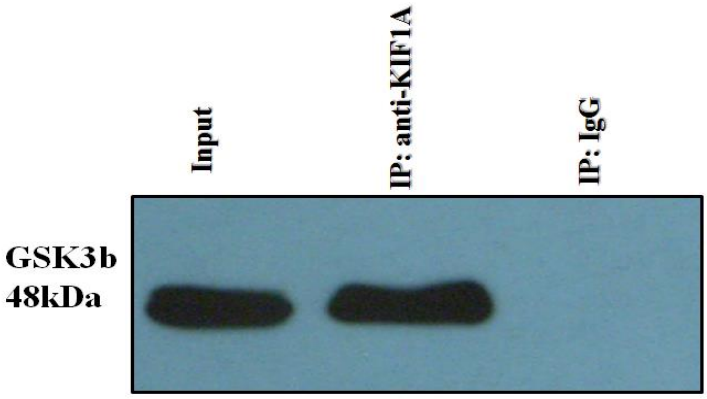


Figure 3-17: KIF1A binds GSK3β in cultured neurons.
A monoclonal antibody to KIF1A immunoprecipitated GSK3β from neuronal lysates showing KIF1A interacts with GSK3β.

A common strategy for motor-cargo binding is that motors may recruits a scaffolding proteins that then binds the cargo or the cargo molecules may interact directly with the motor (Fu and Holzbaur, 2013; Setou et al., 2002). KIF1A and GSK3β may also interact directly or via some intermediate protein. To test this possibility, I immunoprecipitated KIF1A from mouse hippocampal neurons, separated the proteins by SDS-PAGE and performed silver staining and found two distinct bands ~30 and 130 KDa in size (Figure 3-18). I cut these bands out and sent them to University of Victoria for mass spectrometry. The mass spectrometry data was analysed by “Proteome Discoverer” and statistical analyses of the “Proteome Discoverer” result files was performed with the Scaffold Q+S software package. In an effort to link these proteins to vesicle transport, the Allen Brain Atlas (http://www.brain-map.org) was used to look for the expression levels of the proteins identified in the mass spectrometry data (Table 3-8). Most of the proteins found in mass spectrometry data were expressed in very low level in mouse brain and

also they are not linked with transport. This mass spectrometry data does not give a clear notion whether the KIF1A-GSK3β interaction is direct or indirect as KIF1A might interact with GSK3β via a linker protein.

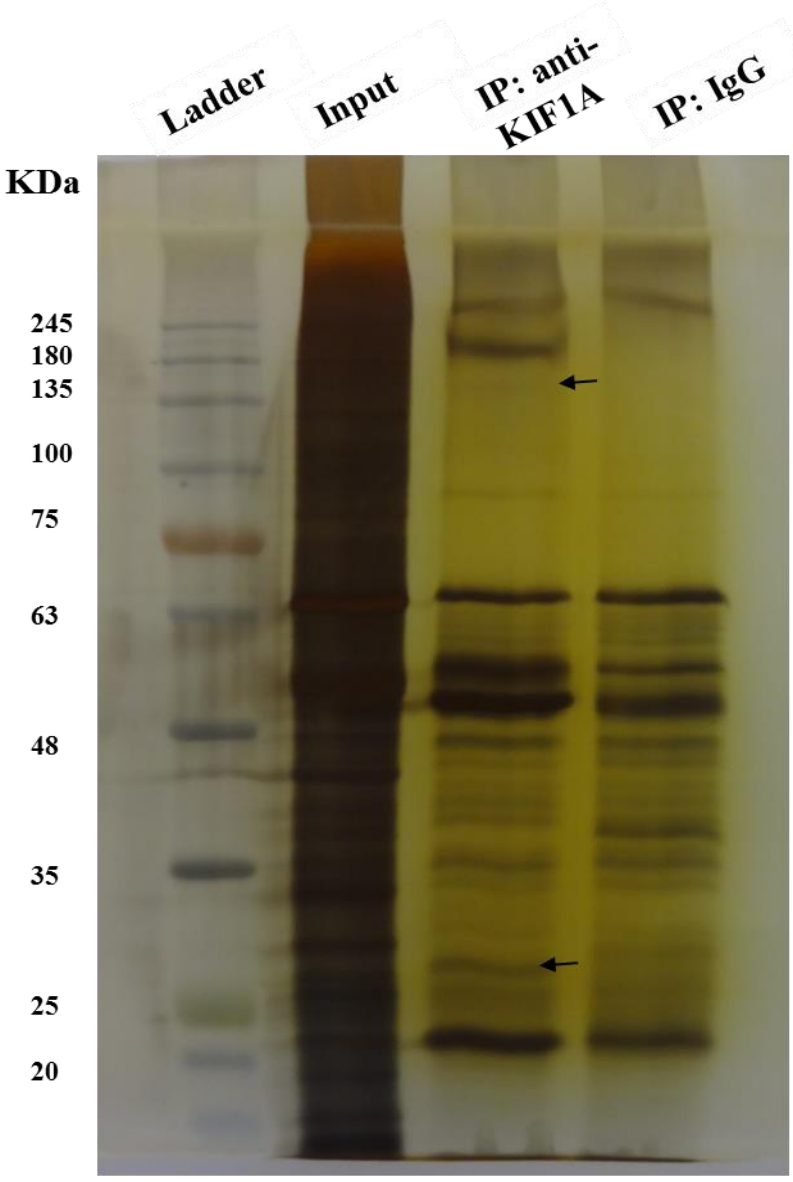


Figure 3-18: Silver stain of immunoprecipitated KIF1A
Silver staining of KIF1A immunoprecipitated samples from mouse hippocampal neurons shows potential protein interacting partners of ~130 and 30 KDa.

Table 3-8: Mass Spectrometry data for potential KIF1A interacting proteins

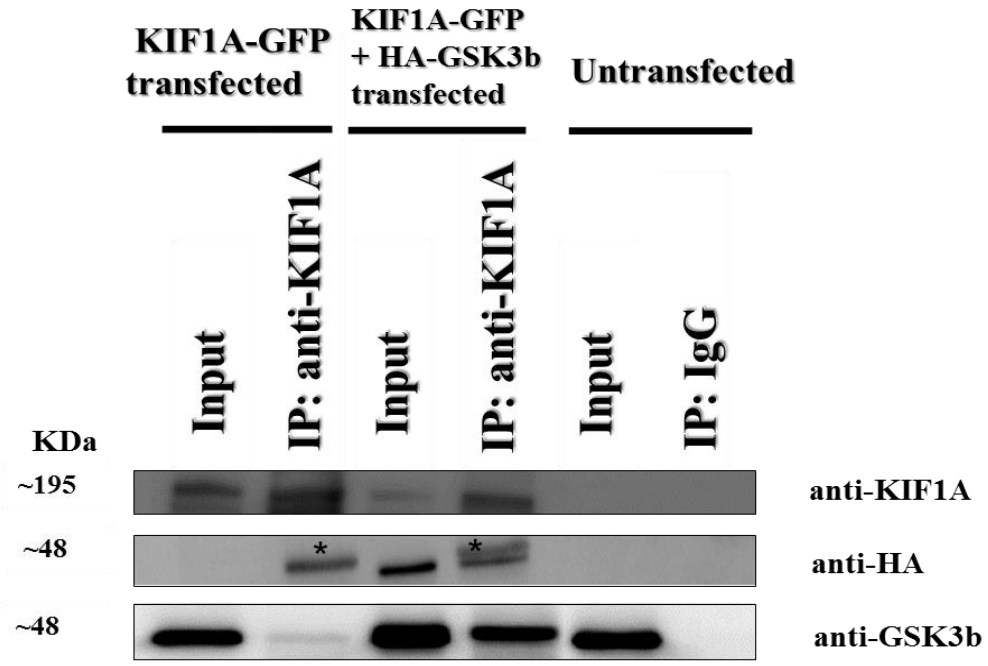
Sample Proteins		Accession No	Protein Identification Probability	Function	Molecular weight
30kDA	130 kDa				
0	100%	B2R4M6	Protein S100	calcium ion binding	13 kDa
0	100%	LYSC_GORGO	calgranulin B	Proinflammatory mediator	17 kDa
0	100%	G3QUU9_GORGO	Lysozyme C	calcium ion binding	11 kDa
0	100%	G1RHF0_NOMLE	Protein S100	microtubule binding,calcium ion binding, zinc ion binding, arachidonic acid binding	11 kDa
100%	78%	Unknown #9684-DECOY	Uncharacterized protein	unknown	?
81%	78%	Unknown #9684-DECOY	Cluster of Unknown decoy protein for peptide	unknown	?
12%	0	Unknown #7501-DECOY	Unknown decoy protein for peptide	unknown	?
0	100%	KIF1A	Kinesin like protein, KIF1A	Motor protein	191 kDa
0	100%	PIP	Prolactin-inducible protein	protein dimerization activity, IgG binding, glycoprotein binding,actin binding	17 kDa

Protein S100 and calgranulin B are expressed in very low levels in olfactory areas and cortical subplate and hippocampus of the brain. Lysozyme C is not expressed in hippocampus and very low concentrations in other areas of the brain such as the medulla and olfactory bulb. PIP is absent while KIF1A is highly expressed in mouse brain (Allen Brain Atlas).

3.6. KIF1A interaction with GSK3β is neuronal cell type specific

To further explore the interaction between KIF1A and GSK3β, I attempted to use a heterologous expression system involving rodent cell lines. These experiments were intended to determine if KIF1A and GSK3β interact directly, and then to map the interaction domains. Initially I carried out experiments in REF52 cells, a rat embryo fibroblast cell line. I expressed HA-tagged GSK3β and GFP-tagged KIF1A in REF52 cells, immunoprecipitated GFP-KIF1A, yet did not detect GSK3β by immunoblotting (See Appendix). This result indicates that the interaction between KIF1A and GSK3β is not direct, but might be via a linker protein that is neuronal cell specific. To test this possibility, I repeated the same experiment in the hippocampal neuronal cell line HT22 and found that KIF1A and GSK3β do interact with each other. These findings show that the GSK3β interaction with KIF1A may be via a scaffolding protein or a post-translational modification present on one or both proteins that is specific to neurons (Figure 3-19 A and B). In the GSK3β immunoprecipitation experiment (Figure 3-19 B), a KIF1A band is absent in the KIF1A-GFP transfected cells and this might be due to the fact that GSK3β is present in high concentrations in the cells and is involved in various different cellular processes like signalling, cell cycle regulation and apoptosis (Grimes and Jope, 2001; Jope and Johnson, 2004; Yang et al., 2006) and the amount of GSK3β which interacts with KIF1A might be relatively low. Future experiments could entail expressing truncated forms of KIF1A and GSK3b in HT22 cells in an attempt to map the sequences required for their interaction.

A) IP KIF1A



B) IP GSK3β

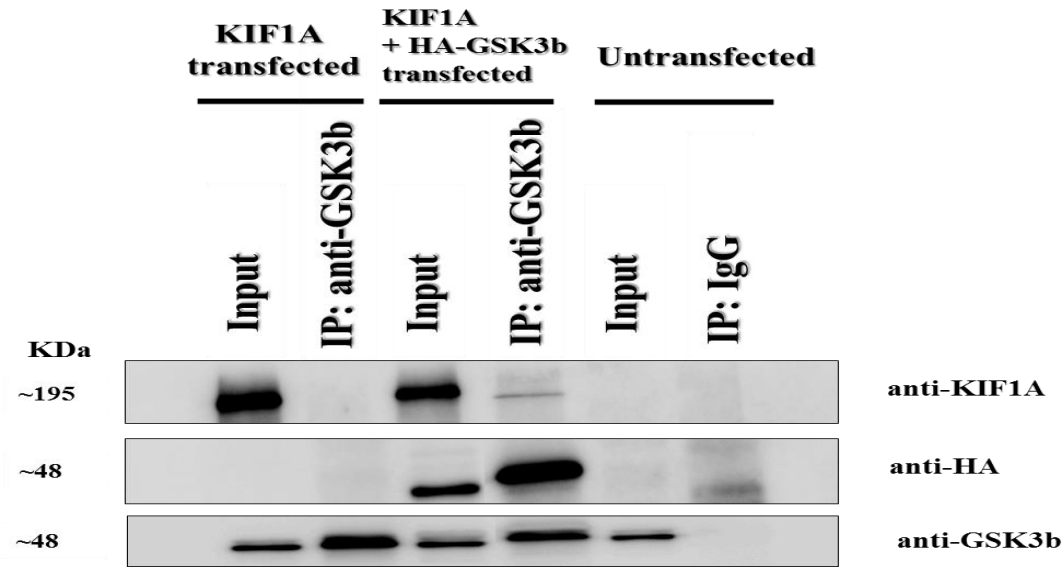


Figure 3-19: KIF1A binds with GSK3β in neuron specific HT22 cell line.
A). Immunoprecipitation of KIF1A from HT22 cells shows that KIF1A interacts with GSK3β (asterisk represents IgG heavy chain). B). The reciprocal immunoprecipitation of GSK3β from HT22 cells shows that GSK3β interacts with KIF1A.

Chapter 4.

Discussion

4.1. Summary

An early predictor of AD is the presence of soluble, non-fibrillar Aβ oligomers (AβOs) that leads to a number of cellular insults including the dysregulation of intracellular signaling cascades, blockade of FAT, synaptic dysfunction, and ultimately cell death (Ferreira and Klein, 2011). FAT disruption is an early pathological event that leads to loss of synapse function and axonal degeneration in AD (Millecamps and Julien, 2013). The mechanism by which motor protein activity and cargo binding are affected in AD are still unclear.

My data show that KIF1A motility in cultured hippocampal neurons from tau $^{+/+}$ and tau $^{-/-}$ mice is reduced in the presence of AβOs and implies that the reduction in flux is likely due to changes in the phosphorylation state of the motor rather than a tau-dependent effect, such as microtubule dissolution. Indeed, tandem mass spectrometry on KIF1A isolated from AD transgenic mouse brain showed that within the putative dimerization domain, Ser 402 is phosphorylated and conforms to GSK3β recognition site. We generated phospho-resistant and phosphomimic forms of KIF1A by point mutation of Ser-to-Ala and Ser-to-Glu, respectively. Live imaging showed that in the presence of AβOs KIF1A (S402A) transport is unaffected while KIF1A (S402E) transport is reduced implying that KIF1A-S402 is critical residue in KIF1A regulation. The data suggests that AβOs impair BDNF FAT via acting directly on KIF1A. Furthermore, coimmunoprecipitation confirmed an interaction between KIF1A and GSK3β in primary neurons and the neuronal cell line, HT22.

4.2. The role of AβOs in inhibiting KIF1A motility

AβOs lead to tau hyperphosphorylation, but the molecular link between tau pathological forms and AβO-mediated FAT defects is unclear (De Felice et al., 2008). My data demonstrates that imaging AβO-treated hippocampal neurons at high temporal and spatial resolution, KIF1A motility is reduced in the absence of tau. Previously, we have imaged only KIF1A cargoes, such as DCVs, and transport reduction in AβO-treated neurons may have resulted from the dissociation of the cargo from the motor. However, my results imply that reduction in the flux of KIF1A is because the motor is affected by AβOs. Motors can be regulated at several steps either by inhibition of motor protein activity by disrupting motor dimerization and/ or preventing the motor-cargo complex from binding microtubules (Figure 4-1) (Verhey and Hammond, 2009; Schlager and Hoogenraad, 2009).

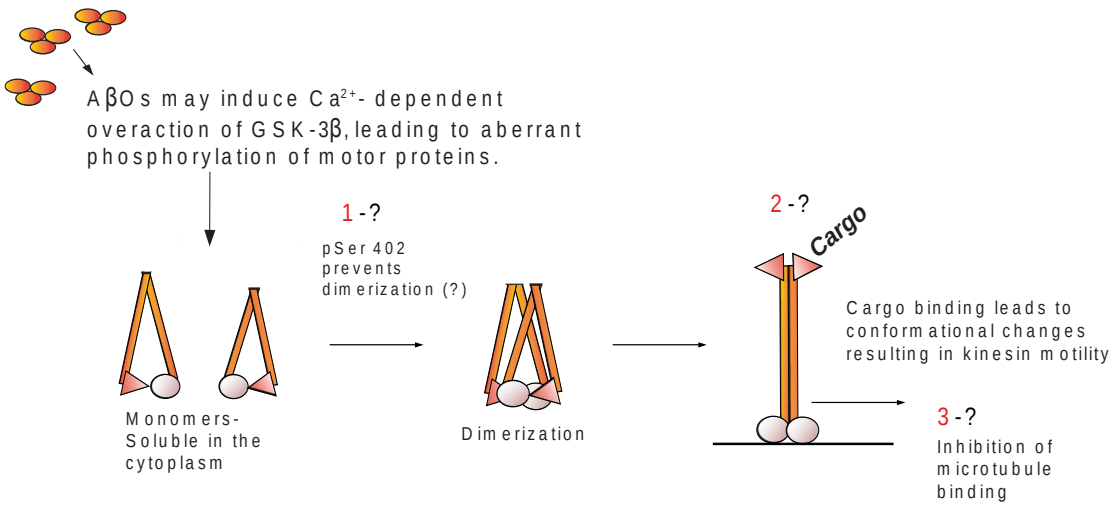


Figure 4-1: Proposed mechanism for KIF1A transport disruption in AD.
At dendrites, AβOs aberrantly activate NMDARs and induce calcium influx, elevating cytosolic calcium. Activated calcineurin relieves inhibition of PP1, which activates GSK3β. GSK3β may inhibit motor protein activity by disrupting motor dimerization (1), via motor-cargo interactions (2), and/or preventing the motor-cargo complex from binding microtubules (3) inhibition of motor binding to microtubules.

Furthermore, previous studies by our lab demonstrate that GSK3β inhibition prevents anterograde and retrograde flux reduction, independent of tau (Ramser and Gan, 2013; Takach et al., 2014). Several studies have shown coordinated regulatory mechanisms by opposing motors in which bidirectional transport is impaired upon disruption of either of two motors (Ally et al., 2009; Uchida et al., 2009; Welte et al., 2009 and Jolly et al., 2011). Hence, this may explain the reduction in flux while other measures such as velocity or run length stay the same. In AD models, bidirectional transport of mitochondria, amyloid precursor protein and organelles contained in squid axoplasm, and primary hippocampal neurons are similarly perturbed (Pigino et al., 2009; Rui et al., 2006; Vossel et al., 2010; Weaver et al., 2013 and Hiruma et al., 2003). Although this phenomenon is commonly observed, subtler changes in axonal transport, e.g., reduction in only anterograde transport, have been detected for different AβO treatments and cargoes (Tang et al., 2012), thus transport regulation can be specific to the motor proteins and cargoes involved.

My data also show that AβOs impair bidirectional transport of KIF1A in the presence of tau, but in tau $^{-/-}$ neurons only anterograde transport is reduced. Hyperphosphorylated tau is generated in neurons exposed to AβOs and tau hyperphosphorylation leads to its detachment from microtubules and exposure of phosphate activating domain (PAD) within the tau N-terminal (Kanaan et al., 2011). The exposure of PAD leads to activation of downstream phosphatases which might specifically inhibit dynein activity. A study by Gao et al., has shown that phosphorylation of dynein by GSK3β leads to reduced dynein motility (Gao et al., 2015). Hence, GSK3 β might be playing a critical role in regulating KIF1A and dynein activity. This can be the possible explanation for reduction of retrograde transport of KIF1A in AβO treated tau $^{+/+}$ neurons. The role of tau on dynein is not well understood, but dynein is a phosphoprotein and impaired retrograde transport is shown to contribute to tau-induced toxicity (Pfister, 2015; Butzlaff et al., 2015).

Some studies support the notion that tau is the main culprit behind transport defects (Ittner et al., 2009; Vossel et al., 2015). Vossel et al., have shown that tau reduction prevented AβO-induced axonal transport defects of mitochondria and TrkA. The difference between my results and this study can be explained by the

difference in mechanism of motor protein regulation. KIF1A carry cargoes like DCVs and SVPs while mitochondria and TrkA are primarly transported by KIF5. Moreover, another difference is that they observed transport deficits after 1 hour of AβO treatment as opposed to 18 hrs of AβO treatment in my study. It is possible that transport defects observed within 1 hour of AβO treatment are due to tau, as high micromolar amounts of AβOs were used which may introduce tau hyperphosphorylation, its detachment from microtubules (Kanaan et al., 2011). On the other hand, I used nanomolar concentration of AβOs where the contribution of pathogenic tau is minimal.

4.3. The putative GSK3β site plays a role in regulating KIF1A motility

My data show that KIF1A is phosphorylated at a conserved GSK3β consensus site (S402) by AβOs. This S402 is present in the dimerization domain of KIF1A and may impair KIF1A motility by two different mechanisms. The aberrant phosphorylation at S402 may prevent KIF1A dimerization and activation or can place a negative charge at S402 that could alter neck coil domain's flexibility and can favor the folded, autoinhibited confirmation of KIF1A. The ionic interactions between the tail and motor domains of KIF5 are indeed stabilized by phosphorylation of S175 within the motor domain, which promotes KIF5 autoinhibition (De Berg et al., 2013). Furthermore, the interaction between the C-terminal domain and neck coil region of kinesin-13 is weakened by Aurora-dependent phosphorylation of S196 which results in opening of its confirmation which leads to reduced affinity of kinesin-13 for microtubules (Ems-McClung et al., 2013). My data show that the transport of the nonphosphorylatable form, KIF1A-S402A, is unaffected in the presence of AβOs (Figure 3.6-3.8 and table 3.2). I expected phosphomimic KIF1A-S402E to have reduced transport, but surprisingly this mutant was still motile in untreated neurons (Figure 3.9- 3.11 and Table 3.3). It implies that the KIF1A-S402E mutant might be interacting with endogenous KIF1A that might dimerize with KIF1A-S402E leading to transport of the mutant. The other possibility is the KIF1A-S402E mutant is bound to the vesicle, and the vesicle is carried by the endogenous KIF1A motor, masking the effect of the mutation. To test this possibility I used RNAi to knock down the endogenous KIF1A, along with expressing an

RNAi-resistant form of KIF1A-S402E. In the absence of endogenous KIF1A, the transport of the phosphomimic KIF1A- S402E is significantly reduced (Figure 3.14-3.15 and Table 3.5) which shows that endogenous KIF1A is influencing KIF1A-S402E motility. On the other hand, in the presence of AβOs, KIF1A-S402E mutant transport is severely reduced and this could be explained by the possibility that KIF1A gets phosphorylated at more than one amino acid and the cumulative effect of phosphorylation at two sites markedly reduced transport.

To test that S402E mutation possibly obstructs KIF1A dimerization and cargo binding, our collaborator (K. Verhey, University of Michigan), is employing a FRET-based strategy. Wild type and mutant KIF1A will be tagged with monomeric versions of FRET donor (mCFP) and acceptor (mCitrine) fluorescent proteins either at the N-terminus to measure the proximity of the motor domains or at the C-terminus to measure the proximity of the cargo binding (tail) domains. The motors will be recruited to the early endosomes, which typically exhibit low motility, by placing the PH domain of KIF16B on the tail of KIF1A (Soppina et al., 2014). KIF1A recruitment to early endosomes and subsequent activation should result in cargo transport to the cell periphery. KIF1A S402A, that is resistant to inhibitory phosphorylation, should behave similarly. In contrast, no FRET signal may be detected for KIF1A-S402E if it indeed fails to dimerize. Hence, we can conclude that KIF1A-S402 is a critical residue in the regulation of KIF1A and KIF1A phosphorylation at S402 can prevent motor dimerization and activation and may impair DCV transport, block cargo binding and reduce motor processivity along microtubules.

4.4. KIF1A-GSK3β interaction is neuronal cell specific

The co-immunoprecipitation experiments showed that KIF1A interacts with GSK3β in a direct or indirect manner (Figure 3.17). Understanding how these two proteins interact can give insight on how GSK3β influences transport. JIP-1b functions as a linker between KIF5 and APP and also enhances the JNK-dependent phosphorylation of APP. So, JIP-1b not only act as a linker but also acts a as a scaffold for JNK signalling, which suggests that axonal transport of KIF5 might be regulated by it (Inomata et al., 2003). The KIF1A and GSK3β interaction can also be facilitated by a scaffolding protein, linker protein

or G-protein and hence can play part in regulating KIF1A transport. The experiments in HT22 cells showed that KIF1A and GSK3β interaction occurs via a protein (Figure 3.19 and 3.20). To identify the protein facilitating KIF1A and GSK3β interaction approaches like SILAC (Stable isotope labelling by amino acids in cell culture) can be used. For example, HT22 cells expressing a GFP-tagged KIF1A can be metabolically labeled by culturing in "heavy" media containing ^{13}C-isotopes of arginine and lysine, while the parental HT22 cells can be grown in "light" media containing the ^{12}C-isotopes of arginine and lysine. Whole cell extracts can be prepared and pre-cleared on Sepharose beads and then mixed in equal amounts before affinity purification of the GFP-tagged KIF1A. KIF1A can be eluted from the beads and separated by SDS-PAGE for digestion and LC-MS/MS analysis can identify KIF1A interacting proteins. KIF1A is a primary anterograde motor (Lo et al., 2011) and GSK3β is a critically important kinase in transport regulation (Mudher et al., 2004; Weaver et al., 2013), thus understanding the KIF1A-GSK3β interaction can help to better understand the mechanisms involved in AD and design compounds for preventing transport defects.

4.5. Conclusion and Future Perspectives

My work is significant because it implicates GSK3β in KIF1A dysregulation in early stages of AD pathogenesis. KIF1A is associated with neurodegenerative diseases (Okamoto et al., 2014), but the phosphorylation dependent mechanisms that lead to KIF1A-cargo interactions and processivity and their contribution to AD have not been investigated. Impaired KIF1A motility might underlie synaptic loss in AD patients and KIF1A is highly expressed in the brain (Kondo et al., 2012), thus compounds can be designed to restore its motility that may exhibit few off-target effects compared to current therapeutics available for AD.

Experiments geared toward therapeutic development should focus on the mechanisms of KIF1A regulation by GSK3β for better understanding of mechanisms underlying AD and axonal transport. Peptide inhibitors can be designed that mimic GSK3β sites on KIF1A and prevent phosphorylation in a dominant positive fashion, hence preventing transport defects. Another approach is to design small molecules that activate KIF1A. Screens for kinesin inhibitors that block cell division are widely used to design

chemotherapeutic agents (Funk et al., 2004). Similar screens to develop allosteric modifiers that maintain KIF1A motility may also be designed. To assure that restoring transport will have a meaningful effect on the disease different cognitive and behavioral tests can be first conducted on AD mice. Understanding the mechanisms of KIF1A regulation can facilitate development of new therapies for AD.

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Appendix

IP KIF1A in REF52 cells

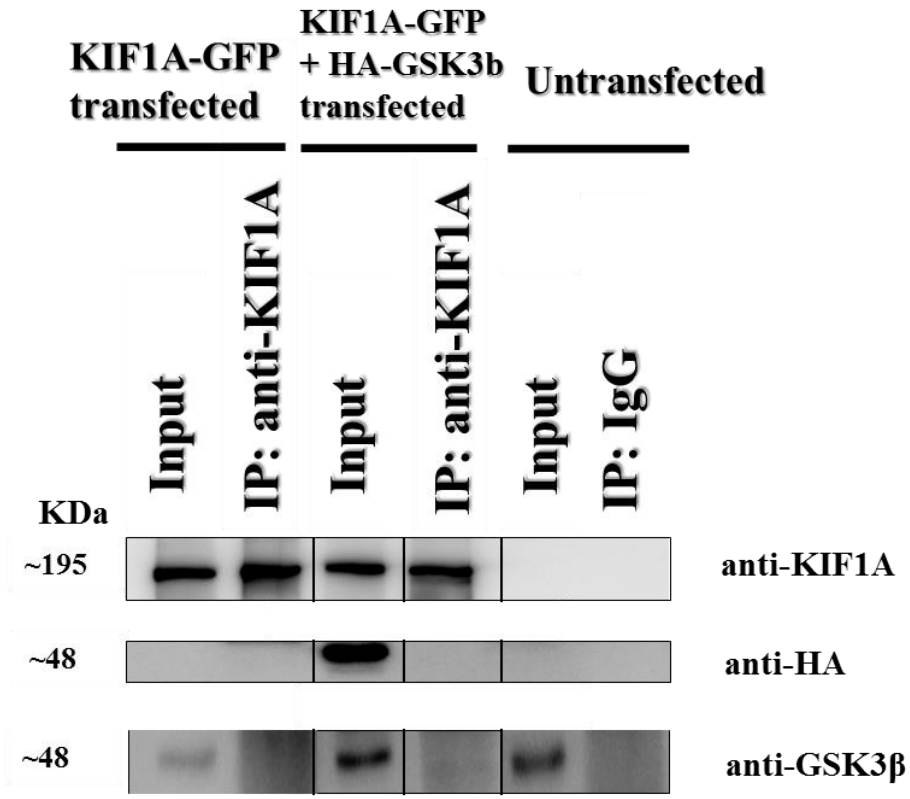

Figure A1: KIF1A does not bind GSK3β in REF52 cells.

Immunoprecipitation shows that KIF1A does not interact with GSK3β in REF52 cells and the KIF1A- GSK3β interaction is likely neuronal cell type specific.