KIF1A: A CANDIDATE MOTOR PROTEIN FOR DENSE-CORE VESICLE TRANSPORT IN CULTURED HIPPOCAMPAL NEURONS

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Anastasia Kuzmin
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Name: **Anastasia Kuzmin**

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Examining Committee:

                Chair: **Dr. Timothy V. Beischlag**

                               Associate Professor of Department of Health Sciences, SFU

Dr. Michael A. Silverman
Senior Supervisor
Associate Professor of Department of Biological Sciences, SFU

Dr. Sherryl Bisgrove
Supervisor
Assistant Professor of Department of Biological Sciences, SFU

Dr. Edgar Young
Supervisor
Assistant Professor of Department of Molecular Biology and Biochemistry, SFU

Dr. Douglas Allan
External Examiner
Assistant Professor of Department of Cellular and Physiological Sciences, UBC

Date Defended/Approved: December 8, 2009

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ABSTRACT

Axonal transport of membrane bound organelles is essential for neuronal cell function and survival. Dense-core vesicles (DCVs) are cargo-carrying organelles in neurons responsible for transporting and secreting neuropeptides such as brain derived neurotrophic factor (BDNF). DCVs are synthesized in the cell body and travel large distances on microtubules to pre- and postsynaptic release sites. This thesis sought to determine if the kinesin-3 family member KIF1A transports DCVs in primary cultured hippocampal neurons. This was done by reducing KIF1A protein expression in neurons through RNA interference and imaging the movement of DCV marker BDNF-RFP in experimental and control cells. Two-colour live-cell imaging of BDNF-RFP and KIF1A-GFP was preformed to establish an association between KIF1A and DCVs. Cells in which KIF1A was reduced showed a marked decrease in overall DCV flux compared to control cells. These data support a primary role for KIF1A in the anterograde transport of DCVs in mammalian neurons.

Keywords: dense-core vesicle, neuropeptide, hippocampal neuron, KIF1A, brain derived neurotrophic factor

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GLOSSARY

ADP	Adenosine di-phosphate
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine tri-phosphate
BDNF	Brain derived neurotrophic factor
CaMKII	Ca2+/calmodulin-dependent Kinase II
CCD	Charge coupled device
CMTA2A	Charcot-Marie-Tooth disease 2A
CPE	carboxypeptidase E
DCV	Dense core vesicle
DIC	Dynein intermediate chain
FHA	Forkhead association domain
GFP	Green fluorescent protein
GRIP1	Glucocorticoid receptor interacting protein
GTP	Guanine tri-phosphate
KHC	Kinesin heavy chain
KIF1A	Kinesin-3 family member 1A.
KIF1Bβ	Kinesin-3 family member 1Bβ
KIF5	Kinesin-1 family member 5
KLC	Kinesin light chain
LSD2	Lipid storage droplet protein 2
LTP	Long term potentiation
MAP	Microtubule associated protein
NCD	Non-claret disjunctional
NGF	Nerve growth factor
NMDA	*N*-methyl *D*-aspartate
NPY	Neuropeptide Y
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
PH	Pleckstrin homology
PI(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PSD	Post-synaptic density
RFP	Red fluorescence protein
RyR	Ryanodine receptors
SV	Synaptic vesicle
SVP	Synaptic vesicle precursor
SNP	Single nucleotide polymorphism
TfR	Transferrin receptor

| TGN | Trans-Golgi network |
| VE-DIC | Video-enhanced differential interference contrast light microscopy |

1: 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Overview

Neurons are remarkable cells, highly specialized to transmit and receive information. This function is ultimately dependent on the polarized distribution ofcellular components, dividing the cell into two major morphologically distinct domains: the axon and the dendrites. These domains are kept morphologically, structurally and biochemically distinct throughout the lifetime of a functioning neuron, primarily by molecular motor proteinsthat transport cytoplasmic components to their appropriate cellular compartments along extensive actin and microtubule networks. Two major classes of molecular motors are responsible for microtubule based cargo transport: kinesins and dyneins. Kinesins move toward the plus end of microtubules while cytoplasmic dynein moves toward the minus end. The appropriate delivery of cargo by these motors is particularly relevant in the case of synaptic function and inter-neuronal communication. Synapses develop in places where axons of one cell contact dendrites of another. They are the conduits through which biochemical and electrical information is relayed between cells.   Biochemical messages are released into the synaptic cleft from either synaptic vesicles (SVs) or large dense core vesicles (DCVs) (Lodish et al., 2008). SVs are formed and filled with classical neurotransmitters at presynaptic release sites where they remain in residence, alternately filling, storing and releasing their contents (Lodish et al., 2008). By

contrast, DCVs travel large distances, from the cell body where the vesicles form, along axons and dendrites to distal pre- and postsynaptic sites where their protein and neuropeptide contents are stored and released (Goldstein and Yang, 2000). DCVs log travel distances of up to 10,000x the diameter of the cell body, underscoring the need for active cytoskeletal-based transport (Goldstein and Yang, 2000). A wide range of biological processsesis facilitated by the contents of these organelles, including neuronal survival, development, learning and memory (Lodish et al., 2008). The mechanism by which these organelles are transported to their release sites is, therefore, an important aspect of proper neuronal communication and function.

A total of 45 kinesins have been identified in the mammalian genome, and motor-cargo interactions are very specific (Hirokawa and Takemura, 2004). Thus, these motors must distinguish among a multitude of cellular cargos for efficient transport. Previous work has shown that the molecular motor, kinesin-3 family member KIF1A transports DCVs in flies and worms (Barkus et al., 2008; Jacob and Kaplan, 2003; Zahn et al., 2004). Through RNA interference mediated knockdown of endogenous protein, this thesis identifies KIF1A, as the primary motor responsible for axonal DCV transport in live rat hippocampal neurons. The study also shows functional evidence of an interaction between KIF1A and DCVs during retrograde axonal transport by evaluating the degree of co-transport of fluorescently tagged KIF1A-GFP and DCV marker BDNF-RFP in axons of live cultured hippocampal neurons. A review of neuronal trafficking is provided with a specific focus on axonal DCV transport and motor-cargo

interactions. Additionally, mechanisms of intracellular bidirectional transport will also be explored.

1.2 Axonal Transport

1.2.1 Neurons

Neurons are polarized cells. Such polarization is reflective of the highly specialized nature of neurons in signal transmission. Neurons consist of a cell body surrounded by dendrites, with one axon emanating from the cell body (Fig. 1). The axon is a long, thin structure that ends in a slightly broadened area that connects with the dendrites of neighbouring cells. Dendrites surround the cell's body and are thicker most proximal to the cell body and taper distally (Lodish et al., 2008) (Fig 1). This type of morphology allows neurons to communicate with each other at specialized sites (synapses) formed by the connection between axon tips and the dendrites of neighbouring cells. Pre-synaptic sites located at axons tips release information in the form of signalling molecules. These are received by the dendrites and the signal is then transmitted through the cell body and passed on to neighbouring cells through the axon.

Underlying the morphological polarization of neurons is the polarization of cytoplasmic components into distinct biochemical and functional domains. One of the most obvious differences between axons and dendrites is that axons lack the protein synthesis machinery present in dendrites that share their cytoplasmic architecture with the cell body (Lodish et al., 2008). As a result all axonal

components must be transported from the cell body into the axon. This presents a challenge for the cell as axon length can be up to 10,000 times the diameter of the cell body (Goldstein and Yang, 2000). Without any mechanisms in place to facilitate cargo delivery to the axon, delivery by diffusion would take so long that the cell would not be able to function. Neurons overcome this problem by the use of active cytoskeletal-based transport.

1.2.2 Axonal cargo and transport

The vast majority of axonal components must be transported there from the cell body (anterograde transport). This includes cytoskeletal components, lipids, organelles as well as signal transduction components. In addition, components destined for degradation must be actively transported from the axons back to the cell body (retrograde transport) (Brown, 2003). This large array of cargos is shuttled along the axon by specialized motor proteins which themselves move along the axonal microtubule network. Axonal cargo can be carried by fast or slow axonal transport. The same families of molecular motors carry out both types of traffic so the difference between them is the manner in which the movement is regulated (Brown, 2003). Cytoskeletal polymers and cytosolic protein complexes are transported by slow axonal transport, at an approximate rate of 0.1 µm / second (Brown, 2003). Their transport is characterized by infrequent short-range anterograde and retrograde movements, resulting in a slow overall gain in distance. Membranous organelles such as synaptic vesicle precursors (SVPs), DCVsand vesicles containing signalling complexes are transported by fast axonal transport in a continuous unidirectional

manner at a rate of 1 – 5 μm / second (Brown, 2003). Mitochondria can move by both fast and slow axonal transport but generally move somewhere in between the two at about 0.2 μm / second (Brown, 2003).

1.2.3 Defects in Axonal Transport and Disease

Neuronal growth, development, maintenance and communication are dependent on proper axonal transport. Therefore, it is not surprising that many neurodegenerative diseases manifest defects in axonal transport. Examples include Huntington's, amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease (CMT) 2A and Alzheimer's (De Vos et al., 2008; Zhao et al., 2001; Stokin and Goldstein, 2006). Huntington's is a familial neurodegenerative disease with symptoms that include loss of memory, inability to make decisions and loss of motor coordination. It is caused by a polyglutamine expansion (above normal number of sequential glutamine amino acids) within the huntingtin protein (De Vos et al., 2008). The mutant huntingtin protein disrupts axonal transport in *Drosophila* as well as mammalian neurons, however the manner in which it does so is not yet understood (De Vos et al., 2008). Lack of proper axonal transport may be one of the contributing factors to the neuronal degeneration characteristic of the disease. CMT 2A is a disease causing peripheral neuropathy. Symptoms include weakness, atrophy of peripheral muscles and mild sensory loss (Zhao et al., 2001). It is caused by a missense mutation resulting in a Q98L sutitution within the ATP binding site of KIF1Bβ, a kinesin motor that has a similar c-terminal domain structure to KIF1A and, like KIF1A transports synaptic vesicles in neuronal axons (Zhao et al., 2001). ALS is a neurodegenerative disease that

causes the degeneration of motor neurons and as a result muscular atrophy. Once again axonal transport appears to be impaired due in part to mutations in the motor-cargo adaptor protein dynactin (Stokin and Goldstein, 2006). Finally, Alzheimer's is a neurodegenerative disease characterized by progressive memory loss and dementia. Importantly, defects in axonal transport are emerging as a hallmark of the disease which manifests itself primarily through protein aggregation and mislocalization (Stokin and Goldstein, 2006).
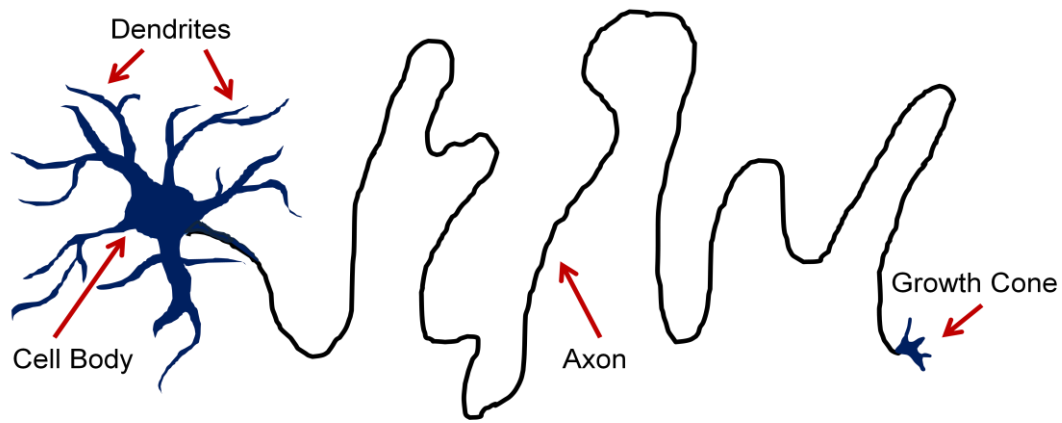
Figure 1. Typical Hippocampal Neuron

The general structure of a hippocampal neuron with dendrites surrounding the cell body and one axon emanating out. The axon ends in a growth cone before it connects with dendrites of other cells. When it does make contact with dendrites of other cells, a synapse is formed.

1.3 Dense Core Vesicles

1.3.1 Biogenesis

Precursors of neuropeptides are synthesised on the rough endoplasmic reticulum, and then packaged into DCVs within the TGN. Dense core vesicles (DCVs), also known as secretory granules, are large vesicles about 100 nm in diameter (Sorra et al., 2006) that bud off the trans-Golgi network (TGN) carrying their neuroactive peptide cargos (Park et al., 2009). From there they are actively transported to dendritic and axonal destinations within the cell. Prior to reaching their sites of release these neuropeptides mature into more processed forms. DCVs mature in conjunction with the neuropeptides they carry.   The process includes acidification, removal of coat proteins and non-DCV associated proteins that failed to sort out within the TGN (Kim et al., 2006). DCV cargos are secreted in response to stimuli and as such are part of the regulated secretory pathway. They must be sorted away from cargos destined for the constitutive secretory pathway that which does not require stimulation for exocytosis (Park et al., 2009). Two models have been proposed to describe this sorting (Borgonovo et al., 2006). The "sorting by entry" model proposes that high calcium concentrations and low pH at specialized locations within the TGN lumen favours the aggregation of DCV cargo. The "sorting by retention" model suggests that cargos of the constitutive secretory pathway are actively removed from immature DCVs. It is likely that both models describe DCV biogenesis (Borgonovo et al., 2006). In support of the "sorting by entry" model, the aggregation of the DCV cargos, granins and prohormones at lipid rafts within the TGN seems to drive

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DCV budding from the TGN (Kim et al., 2006). The "sorting by retention" model seems to describe the maturation process of DCVs, where vesicles containing proteins inadvertently packaged with the DCVs are constitutively budded off (Kim et al., 2006).

1.3.2 DCV Transport and Release

After budding off from the TGN maturing DCVs are transported along microtubules by motor proteins of the kinesin and dynein families to sites within the dendrites and the axon. In flies and worms the kinesin motor responsible for the anterograde axonal transport of DCVs is *UNC-104*, a homolog of the mammalian kinesin KIF1A (Barkus et al., 2008; Jacob and Kaplan, 2003). Once DCVs reach the axon terminal they are either released through the "ready releasable pool" in response to stimulation or they join the reserve pool (Burgoyne and Morgan, 2003). Studies of *Drosophila* neuro-muscular junctions indicate the reserve pool consists of a mobile population of DCVs that move throughout the axon and resting nerve terminals. In response to stimulation these mobile DCVs are captured at the axon terminals and their contents are released via exocytosis. Indeed, the researchers did not detect an increase in DCV traffic from the soma but rather an increase in the capture of transiting DCVs at nerve terminals in response to signalling, a phenomenon they refer to as activity-dependent synaptic capture (Shakiryanova et al., 2006). The same group later showed DCVs exhibited increased mobility at the synapse in response to Ca^{2+} influx. They went on to show that Ca^{2+} release through the ryanodine receptors (RyRs) of the smooth ER in axon terminals activated

Ca2+/calmodulin-dependent Kinase II (CaMKII) that in turn produced a lasting mobilization of DCVs (Shakiryanova et al., 2007). These studies demonstrate that DCVs are not simply recruited from the soma in response to signalling which in some cases can take a long time to deliver even with fast axonal transport. It seems that an overabundance of DCVs are shipped from the cell body and circulated through axon terminals ready to be recruited quickly when needed (Shakiryanova et al., 2007). DCVs that are captured for release are docked at the plasma membrane, primed and then fuse with the axon terminal membrane (Burgoyne and Morgan, 2003).

The fusion can either be complete where the entire contents of the DCV are released into the synaptic cleft or it can be partial. Partial fusion, often called "kiss and run" involves a rapid reclosure of the fusion pore so that the granule is not completely emptied (Burgoyne and Morgan, 2003). One study in rat sympathetic cervical ganglia indicates that these partially emptied DCVs are transported back to the cell body by fast axonal retrograde transport. The authors hypothesize that these vesicles and their components are either recycled within the cell body or the vesicles are refilled with neuropeptide cargos from the TGN (Li and Dahlström, 2007). Thus, axonal transport is not only required for the delivery of DCVs to axon terminals from the soma but also for the circulation and mobilization of the reserve pool.

1.4 DCV Cargos

Peptides carried within DCVs such as hormones and neurotrophins can have profound influences on the organism as a whole as their release and action

influences nearly every aspect of physiology. Neurotrophins constitute a class of neurotransmitters that influence cell survival, differentiation and growth (Lodish et al., 2008). They consist of the neuropeptides neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) which interact specifically with protein kinase receptors of the *trk* family (Lodish et al., 2008). BDNF is highly expressed throughout the brain, including the hippocampus where it promotes cell survival (Marmigère et al., 2003). It is an abundant component of DCVs and was used as a DCV marker in this study. Within the hippocampus, BDNF causes long lasting changes to neuronal connectivity by enhancing the growth of dendritic spines and thereby increasing synaptic density (Lochner et al., 2006). In hippocampal neurons the building of new synaptic sites is associated with increased long term potentiation (LTP), a phenomenon that is responsible for increased memory and learning (Bekinschtein et al., 2008). BDNF enhances LTP by causing the transcription of genes that are involved in building synapses (Bekinschtein et al., 2008).   As a result, BDNF knockout mice have severe defects in long-term memory (Lu et al.,2005) and *trkB* (BDNF receptor) knockout mice have a decrease in spine density (Tyler and Pozzo-Miller, 2001). A very interesting finding was that people who had a single nucleotide polymorphism (SNP) within the pro- region of BDNF (one of the unprocessed forms) had impaired hippocampal based episodic memory. Importantly this study showed that in cultured hippocampal neurons this mutation caused impairment of BDNF trafficking to distal dendrites and

axons (Egan et al., 2003). Reduced BDNF levels are also associated with depression (Shieh et. al., 2008) and Huntington's disease (Gauthier et al., 2004).

The neuroendocrine hormone neuropeptide Y (NPY), another DCV peptide, is moderately expressed in the hippocampus (Lindner et al., 2008) and was also used to image DCVs in this study. It plays a major role in regulating food consumption, energy homeostasis, stress, blood pressure, memory retention as well as other processes (Lin et. al., 2004). In spite of the seemingly many functions of NPY the mouse knockout phenotype is relatively mild. NPY deficient mice are viable and fertile, have no overt abnormalities and have normal food intake (Thorsell and Heilig, 2002). They do however, appear to have increased anxiety, can be prone to seizures and can be less sensitive to the effects of alcohol (Thorsell and Heilig, 2002). Transgenic rats overexpressing NPY demonstrated a loss of memory acquisition (Thorsell and Heilig, 2002). A stronger affect was seen with exogenous application of NPY, which led to a reduction in anxiety, increased food intake, increased vasoconstriction and dosage dependent changes in memory acquisition (Thorsell and Heilig, 2002). Clearly, then, axonal transport of such important neuropeptides is fundamental to proper neuronal function. These important DCV cargos rely on the motor proteins to deliver them to their destinations, yet the molecular motors responsible for their axonal transport have only been extensively explored in invertebrates (Jacob and Kaplan, 2003; Zahn et al., 2004; Barkus et al., 2008).

1.5 Microtubule-Based Motors

Kinesins and dyneins are two superfamilies of molecular motor proteins with a wide range of activities including microtubule based organelle transport (Hirokawa and Takemura, 2004) and mitotic spindle dynamics (Scholey et al., 2003). Importantly, they are also responsible for active cytoskeletal based cargo transport where they transport cargos along the neuronal microtubule network. Microtubules are composed of heterodimers of α- and β-tubulins, which assemble to form protofilaments. These then associate with each other laterally to form the tube like microtubules. The tip of the protofilament that contains available β-tubulin for binding is the plus end while the other tip, which contains α-tubulin available for binding is the minus end. Microtubules are highly dynamic, often cycling between states of polymerization and depolymerization. The rate of polymerization and depolymerization proceeds much faster at the plus end than it does at the minus end (Lodish et al., 2008). These features give microtubules polarity, which in turn enables the vectorial transport achieved by molecular motors. In neurons, dendritic microtubules can have either plus or minus ends proximal to the cell body (mixed polarity) while in axons the microtubules have a uniform polarity with their plus ends toward the axon tip (plus end out) (Goldstein and Yang, 2000).

Both kinesins and dyneins are ATPases that have a microtubule binding domain and a cargo- binding domain (Fig. 2). Kinesins generally move toward the plus end of microtubules, though there are exceptions, and dyneins move toward the minus end. The existence of axonemal dyneins has been known

since the 60s but cytoplasmic dynein and kinesins were only discovered in the mid 80s (Paschal et al., 1987; Vale et al., 1985; Brady, 1985). Two landmark papers published in 1985 marked the momentous discovery of kinesin. One paper by Vale et al., (1985) purified kinesin from squid axoplasm and bovine brain and for the first time characterized its movement. The other paper, by Brady (1985), purified kinesin from chick brains and demonstrated that it was an ATPase, which associated with microtubules (Brady, 1985). Indeed it was the development of video-enhanced differential interference contrast light microscopy (VE-DIC) in the early 80s which provided the substantial gain in image contrast needed to visualize and measure kinesin based motility (Salmon, 1995). In neurons the plus end out arrangement of microtubules within the axon stipulates that kinesins carry cargo toward the axon tip while dyneins carry cargo toward the cell body.
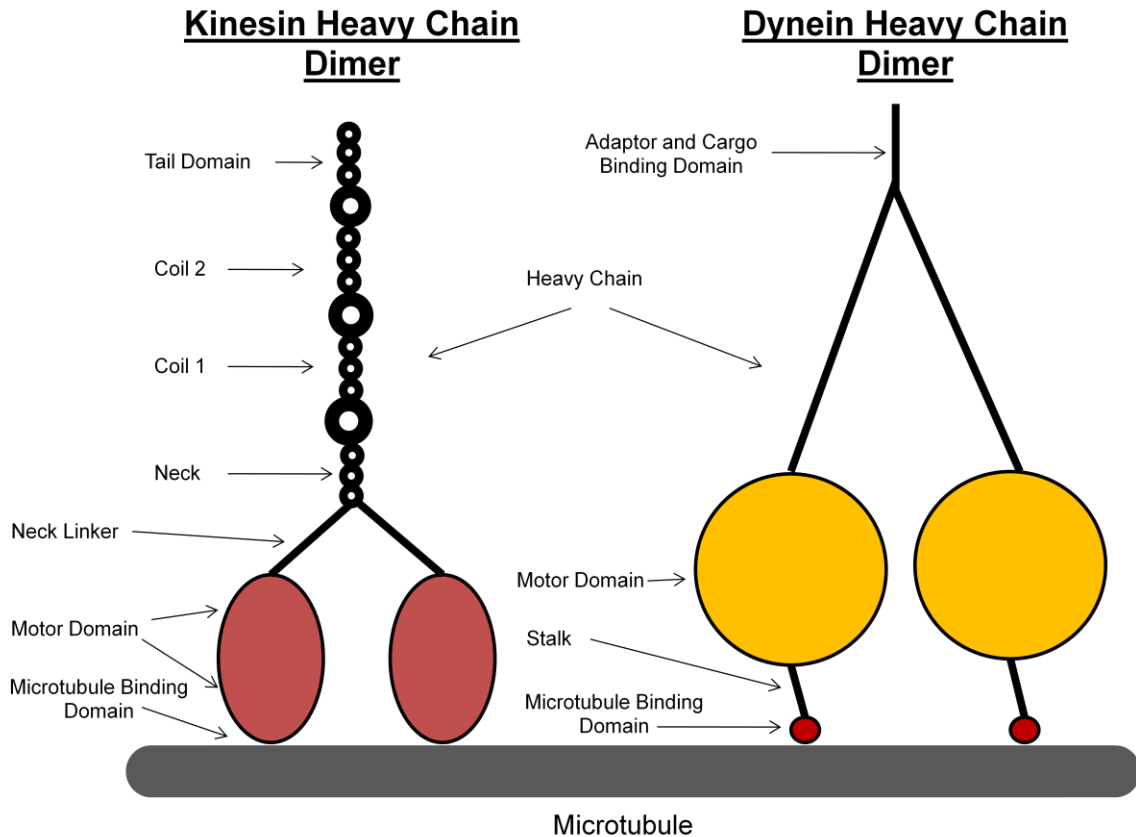
Figure 2. The Two Main Families of Microtubule-Based Molecular Motors

Domain structure of conventional kinesin (kinesin 1) and dynein. Two heavy chains of conventional kinesin, which consist of a motor and microtubule binding domain, a stalk domain that associates with another heavy chain monomer and a cargo binding domain, associate to form a dimer. The N-terminal of kinesins contains the motor domain which also harbours the microtubule binding sites and the ATP hydrolyzing sites. The C-terminal contains the tail domain which binds cargos either directly or through adaptor proteins. Two cytoplasmic dynein heavy chains are shown in association. Each heavy chain has a microtubule binding domain at the C-terminus connected by a coiled coil stalk to the motor domain. The cargo and adaptor binding region is at the N-terminus.

1.6 Dynein

In contrast to the numerous kinesin families there is only one form of

cytoplasmic dynein that transports organelles and is also involved in mitosis

(Vale, 2003). The other form of dynein (axonemal dynein) is generally

associated with the function of cilia and flagella (Vale, 2003). Dynein consists of

a heavy chain with a motor domain, a microtubule binding domain and a cargo-

binding domain. The dynein motor domain is huge, because it contains six AAA

ATPase domains in tandem arranged as a ring. The first four domains bind ATP

although it seems that the first domain is the most important. The last two

domains have a structural role. Globular domains at the tip of coiled coil stalks,

which protrude from the motor domain, bind microtubules. The N-terminus of the

dynein heavy chain contains the cargo and adaptor protein-binding domain (Vale,

2003) (Fig. 2). Although dynein usually exists as a dimer of two heavy chains, it

does not move in a hand over hand motion like kinesin. This is in part because

its microtubule-binding domain is separated from its ATP hydrolyzing domain

whereas in kinesins and the actin-based motors, myosins, they are very close.

Instead, complex conformational changes not yet clearly understood direct

dynein movement toward the minus end of microtubules (Kon et al., 2009).

Since cytoplasmic dynein is the dominant microtubule minus end directed motor

in neurons it conducts all retrograde transport within the axon. It is therefore the

most likely candidate for the axonal retrograde transport of DCVs. This in itself

brings up the interesting question of how a minus end directed motor got down to

the axon tip in the first place? In order for dynein to be able to transport cargo

from the axon tip to the cell body it must itself be transported to the tip which

would necessitate the involvement of kinesin motors. How this is done is not yet

clearly understood, however the current models (discussed later in this thesis)

suggest that it hitches a ride on anterogradely moving vesicles.

1.7 Kinesins

1.7.1 Family Members

All kinesin superfamily proteins (KIFs) have a motor domain that binds and hydrolyzes ATP. All of them fall into three different classes based on the location of the motor domain within the protein. KIFs with a motor domain at their N-terminal (N-kinesins) travel toward the plus end of microtubules. Kinesin families 1-8 and kinesin-11 are all N-kinesins. M-kinesins have a motor domain in the centre of the protein and also move toward the plus end of microtubules however unlike the other kinesins, M-kinesins can depolymerise microtubules. C-kinesins have their motor domain at the C-terminus of the protein and these kinesins move toward the minus end of microtubules (Miki et al., 2005). KIF1A belongs to the kinesin-3 family and as such contains its motor domain at the N-terminus and its cargo binding region at the C-terminus (Miki et al., 2005). All kinesins are composed of a heavy chain that consists of a head domain containing an ATP binding region and a microtubule binding region, a stalk which is composed of coiled coils and contains various hinge regions and a tail domain which is involved in binding cargo or various adaptor proteins (Woehlke and Schliwa, 2000) (Fig. 2). Motor domains are separated from the stalk by a neck linker region that allows the motor domains to move freely in a step-wise fashion (Yildiz and Selvin, 2004).

1.7.2 Kinesin Movement

When kinesins are not working they exist in a soluble, autoinhibited state within the cytoplasm and are not bound to cargo or microtubules (Reilein et al., 2001). Upon cargo-binding the autoinhibitory state is released (Reilein et al., 2001; Lee et al., 2004). The two head domains of a kinesin heavy chain dimer move stepwise along microtubules very much like human bipedal movement. The ATPase within the kinesin motor domain is bound to ADP when it is not bound to a microtubule. Upon motor binding to the microtubule ADP is released and ATP is bound and then hydrolysed to ADP to produce the power stroke. The head then detaches from the microtubule in the ADP bound state. Thus one cycle of ATP hydrolysis corresponds to one step. Conformational changes within the motor domain induced by ATP binding cause the coupling and uncoupling of kinesins to tubulin. In the presence of ATP kinesin binds to both α- and β-tubulin, spanning the cleft between the two monomers (Amos and Hirose, 2007; Mizuno et al., 2004). The dimerised motor thus moves along the microtubule in a hand over hand motion as each of the head domains takes turns binding and releasing from the microtubules (Amos and Hirose, 2007). As a result kinesin step sizes are 8 nm, exactly the length of space that the motor domain covers when bound to α- and β-tubulin (Hancock and Howard, 1999). The main determinants of kinesin directionality are the conformational changes to the neck and linker regions located between the stalk and the motor domains. The involvement of the neck linker in establishing kinesin directionality was definitively shown through chimeric kinesin experiments (Sablin, 2000). Non-claret disjunctional (ncd) protein is a microtubule minus end directed motor that is very similar to

kinesin within its motor domain but not its linker domain. When a kinesin motor domain was fused to an ncd linker domain the chimeric motor moved toward the minus end of microtubules. When the reverse was done with the ncd motor fused to a kinesin linker domain the chimera travelled toward the plus end of microtubules (Sablin, 2000).   Kinesins are considered to be high velocity motors even though the velocity with which kinesins travel varies between family members with some travelling at speeds as low as 0.3 µm/second (KIF3A/KIF3B heterodimers) or as high as 1.5 µm/second (KIF1A) (Hirokawa, 1998). Kinesins are also highly processive and can travel tens of micrometers without falling off the microtubules (Toprak et al., 2009). This feature is particularly useful in axonal transport where great distances must be traversed as quickly as possible.

1.7.3 Cellular Functions

All KIFs share a relatively high sequence homology within their motor domains but are very divergent at their cargo binding ends. As a result they can bind numerous cargos which is reflected in the diversity of their cellular functions. In many cases a single motor can transport a variety of cargos or an individual cargo can be transported by multiple motors (Table 1). In mice, rats and humans 45 different KIFs are coded for in the genome (Hirokawa and Takemura, 2004). By far the majority of these kinesins are involved in mitosis and meiosis and a slightly fewer number are involved in organelle transport (Hirokawa and Takemura, 2004). Polarized cells such as neurons provide an excellent system in which to study motor based organelle transport due to the extensive transport

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networks needed to deliver components to the axons and dendrites (Miki et al., 2005).

The first kinesin was discovered in neurons and termed kinesin heavy chain (KHC). It belongs to the kinesin-1 family, which includes the animal KHC homologues KIF5A, KIF5B and KIF5C. They transport a variety of membrane bound vesicles and mitochondria and act in Golgi positioning (Wozniak et al., 2004). Kinesin-2 family members also transport organelles. Members of the kinesin-2 family include KIF3A, KIF3B/C and KIF 17. KIF3 has been extensively studied through its transport of *Xenopus* melanophores (pigment organelles). KIF3 also transports proteins into cilia and flagella (Deacon et al., 2003). Kinesin-2 family members have not yet been found in fungi or higher plants, which may reflect their dominant function in cilia and flagella (Miki et al., 2005). KIF17 is a neuronal dendritic motor that transports N-methyl D-aspartate (NMDA) receptor subunits to dendritic spines (Hirokawa and Takemura, 2004). Unlike members of the two preceding families, which are dimers, members of the kinesin-3 family can exist as monomers or homodimers. The neuronal specific family members KIF1A/Unc104 and KIF1B have both been implicated in axonal traffic of synaptic vesicles. Although they are not splicing variants, KIF1B is very similar to KIF1A particularly in its cargo-binding domain, which is normally quite divergent between kinesins (Hirokawa and Takemura, 2005). Kinesin families-4 through-14 predominantly function in mitosis and cytokinesis. Well known members include Eg5 from the kinesin-5 family and CENP-E of the kinesin-7 family. Eg5 is crucial for establishing the bipolar spindle and the microtubule

interactions that drive spindle pole separation. CENP-E anchors the

chromosomes to the mitotic spindle and crosslinks the overlapping microtubules

(Wozniak et al., 2004).

Neuronal Cargos Transported by KIFs

Axonal Transport

KIF	Cargo
KIF1A	Synaptic vesicel precursors
	Dense-core vesicles*
	Liprin-α
KIF1Bβ	Synaptic vesicle precursors
KIF1Bα	Mitochondria
KIF5A	Neurofilaments
KIF5	Tubulin dimers
	Mitochondria
	APP
	ApoER2
	JIP
	Huntingtin-associated protein 1
KIF3A/3B	Fodrin
	Par3
	N-cadherin
	β-catenin

Dendritic Transport

KIF	Cargo
KIF1A	GRIP1**
KIF17	NR2B
KIF5	GRIP1
	GluR2
	mRNP complex

Table 1. KIF Motors and Their Various Cargos

Many different kinesins are expressed in neurons and they transport numerous cargos. The table shows examples of cargos carried by different kinesin family members into axons or into dendrites. In some cases the same motor can carry different cargos (KIF5, KIF1A). In other cases the same cargo can be carried by different motors (mitochondria; KIF5 and KIF1Bα, synaptic vesicle precursors; KIF1A and KIF1Bβ). Adapted from *Hirokawa and Noda, 2008*, *Jacob and Kaplan, 2003; Barkus et al., 2008*, ** *Shin et al., 2003*.

1.8 KIF1A

1.8.1 Discovery

Hall and Hedgecock first discovered the kinesin-3 family member KIF1A in 1991 through their work with *Caenorhabditis elegans* mutants. They found that when the gene, which they called *UNC-104*, was mutated the animals displayed various degrees of paralysis due to the improper formation of cholinergic synapses and neuromuscular junctions. Notably, they detected defects in the axonal transport of SVPs leading to a decrease of the accumulation of these vesicles at presynaptic terminals and an increase in their accumulation within the cell body (Hall and Hedgecock, 1991). Later, Okada et al., (1995) isolated the *UNC-104* homolog KIF1A from murine brain and demonstrated that it also transports SVPs. Importantly they found that it was the fastest and most processive, anterograde axonal transport motor travelling at speeds up to 1.5 µm/s. They hypothesised that KIF1A is monomeric because unlike other kinesins discovered so far, KIF1A lacked the long coiled coil domain domains typical of dimeric kinesins (Okada et al., 1995). Since then KIF1A/*UNC-104* mediated axonal transport of SVPs has been extensively studied in vertebrate (Okada et al., 1995; Yonekawa et al., 1998) and invertebrate models (Hall and Hedgecock, 1991; Klopfenstein and Vale, 2004; Pack-Chung et. al., 2007) where it was also shown to transport DCVs (Jacob and Kaplan, 2003; Barkus et al., 2008; Zahn et al., 2004).

1.8.2 KIF1A Cargo

The most definitive evidence for KIF1A mediated transport of SVPs in vertebrates came from mouse knockout studies. The lack of KIF1A led to a phenotype similar to that of *UNC-104* deficient worms in that the mice displayed improperly developed presynaptic nerve terminals and the accumulation of synaptic vesicles in the cell body and axon tips. Unlike the worms, KIF1A deficient mice died shortly after birth, due to massive degeneration of neurons in the central nervous system, specifically the hippocampus (Yonekawa et al., 1998). The authors hypothesise that this may be due to the near lack of neurotransmission at the synapses of mutant mice, which is rescued when mutant neurons are co-cultured with wild-type neurons or with glutamate (SV cargo). It is possible that the neuronal degeneration observed by the authors was the result of a lack of KIF1A mediated DCV trafficking. The observed neurodegeneration is consistent with the fact that DCVs carry neuropeptides, which promote neuronal survival and growth. The authors did not look for an accumulation of DCVs in the cell body, a feature that was observed in other studies where KIF1A was mutated (Zahn et al., 2004; Jacob and Kaplan, 2003).

Several years later researchers showed that *UNC-104* is indeed responsible for axonal transport of DCVs in *C. elegans*. In contrast to the previous study, they did find an accumulation of DCVs in the cell body as well as in the axon tips of *UNC-104* mutants (Jacob and Kaplan, 2003). The results of this research were supported the subsequent year when intracellular transport dynamics of DCVs were examined in *C. elegans Unc-104* mutants (Zahn et al.,

2004). Interestingly, *UNC-104* mutants do not show the neuronal degeneration observed in vertebrate mutants, probably a result of species difference. Even more recently KIF1A was shown to transport DCVs in *Drosophila* (Barkus et al., 2008)*.* Lack of KIF1A causes lethality during the instar stages so adult flies do not develop. Mutant larva displayed uncoordinated movement as well as accumulation of DCVs in axon terminals (Barkus et al., 2008).

Still missing is functional evidence for the transport of DCVs by KIF1A in mammalian neurons. One recent study co-precipitated KIF1A with carboxypeptidase E (CPE), a DCV associated peptide implicated in sorting BDNF to DCVs. However, no *in vivo* transport association between KIF1A and DCVs was explored by this study (Park et al., 2008). Based on the evidence that KIF1A carries SVPs in vertebrates and invertebrates and that KIF1A has also been shown to associate with DCVs in invertebrates, it is very likely DCVs may constitute an additional KIF1A cargo in vertebrates as well. The ability of a single motor to transport different cargos is widespread among kinesins and often involves a variety of cargo-specific linker proteins (Hirokawa and Noda, 2007).

1.9 Motor-Cargo Adaptor Proteins

1.9.1 Kinesin Cargo Linkers

The diversity of cargos required to be transported to specific sites by dynein and kinesin motors in neurons underscores the need for motor cargo specificity. In neurons certain cellular components clearly localize to either axons or dendrites while others are present throughout. For instance, SVs (Yonekawa

et al., 1998) and the microtubule associated protein (MAP), tau (Maganani et al., 2007), are localized to the axon. The transferrin recptor (TfR) (Silverman et al., 2001) and MAP2 (Maganani et al., 2007) are localized almost exclusively to dendrites. Other cargos such as mitochondria (Ligon and Stewart, 2000) and DCVs (Kwinter et al., 2009) are present throughout the cell. How do motors distinguish between different cargos and different cellular locations? In other words how do they achieve such a sophisticated level of vectorial transport? Motor-cargo interactions can be very complex as motors can bind cargos directly or through of numerous adaptor proteins. Emerging evidence seems to show that in many cases these adaptor proteins are also scaffolding proteins, which can bind multiple proteins. These scaffolding molecules are necessary for organizing signalling modules into concrete units of function at specific sites within the neuron (Schnapp, 2003). Understanding how kinesins recognize, bind and transport cargo is not only important for understanding normal brain function but also improper neuronal trafficking.

1.9.2 Motors, Adaptor Proteins and Transport Specificity

Although motors and their interactions with cargos have been extensively studied, it is still not clear how motor-cargo binding and transport are coordinated. It has been proposed that transport can be dependent on the interpretation of local cues by the motor (smart motor), as well as the reverse where local cues act on the cargo to direct the motor-cargo complex to the correct compartment without the active participation of the motor (dumb motor) (Guzik and Goldstein, 2004). The current data suggests that proper transport is

dependent on motors and cargos and more specifically the interaction between them. Both KIF5 and KIF1A can bind multiple cargos that bind to different regions of the motor. KIF5 binds glucocorticoid receptor interacting protein 1 (GRIP1) associated dendritic cargos through amino acids 807-934 (Hirokawa and Takemura, 2005) and kinesin light chain (KLC) associated axonal cargos through amino acids 771-813 (Wozniak and Allan, 2006). KIF1A binds liprin alpha associated GRIP1 cargos through amino acids 657-1105 (Shin et al., 2003) and SVPs through its more c-terminal PH domain (Klopfenstein and Vale, 2004). The over-expression of various cargos that bind different regions of KIF5 in neurons, did not result in changes in the amount and localization of other KIF5 cargos, indicating that cargo-motor binding is non-competitive (Hammond et al., 2008). Kinesin cargo binding domains contain a number of varying protein-protein or protein-lipid binding regions. It is this variation that reflects the ability of single motors to bind multiple cargos.

Cargos bound by molecular motors often comprise entire protein complexes. GRIP1 is a scaffolding protein that itself associates with numerous other proteins involved in synaptic development (Gindhart, 2006). Kinesin light chains also mediate the attachment of KIF5 to entire protein complexes such as members of the JNK signalling cascade (Koushika, 2008). In fact several studies have shown that entire pre-assembled protein complexes are transported into axons (Tao-Cheng, 2007; Gindhart, 2006; Koushika, 2008). This allows kinesin to transport a larger number of cargos and facilitate the localization of signal transduction components (Verhey and Rapoport, 2001). The transport of entire

pre-assembled protein complexes is particularly relevant in axonal transport as it would eliminate the inefficiency and timeliness of transporting components individually. What has become clear is the localization of signalling components, especially within neurons, must be tightly controlled and is dependent on transport. Elucidation of the nature of motor-cargo transport specificity will lay a biochemical foundation for behaviour, learning as well as neurodegeneration.

1.9.3 KIF1A and Cargo Binding

An interaction between KIF1A and GRIP1 has been demonstrated in *Drosophila* where it is also mediated by liprin-α (Spangler and Hoogenraad, 2007). In hippocampal neurons KIF1A amino acids 657-1105 bind GRIP1 associated liprin-α (Shin et al., 2003). Although KIF1A and liprin α colocalize in both dendrites and axons the association of this complex with GRIP1 was limited to dendrites of hippocampal neurons (Shin et al., 2003). Other studies have shown that GRIP1 is a dendrite specific protein (Setou et al., 2002) so it is interesting to speculate that GRIP1 may contain intrinsic properties that prevent the proteins it associates with, including motors, from entering axons. GRIP1 associates with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR2 and numerous other AMPA associated proteins and scaffolds them within the post synaptic density (PSDs) of dendritic spines (Gindhart, 2006). Thus GRIP1 and the proteins it scaffolds may associate with two different kinesin motors, KIF1A and KIF5. This is not surprising due to the importance of such cargos to basic neuronal function. The cells would have backup systems in the case of malfunction of one delivery system.

Similar to KIF5, KIF1A delivers cargos to both axons and dendrites.

Studies of synaptic vesicle transport in *C. elegans*, demonstrated that specific

amino acids within the C-terminal Plekstrin Homology (PH) domain of KIF1A bind

to the membrane phosholipid, phosphatidylinositol-4,5-bisphosphate PI(4,5)P2

and that this binding is necessary for axonal transport of SVPs (Klopfenstein and

Vale, 2004). PI(4,5)P2 is an acidic phospholipid which associates with lipid rafts.

Lipid rafts are microdomains found within plasma membranes that are enriched

in cholesterol, unsaturated fatty acid chain containing lipids and protein

complexes. As a result PI(4,5)P2 containing lipid rafts have important roles in

cell signalling and membrane trafficking, in addition to actin cytoskeleton

regulation (Caroni, 2001).   In addition PI(4,5)P2 interacts with synaptic vesicle

associated proteins and regulates synaptic vessicle endo- and exocytosis

(Rohrbough and Broadie, 2005). Although the entire PH domain of KIF1A is

necessary for binding PI(4,5)P2, argenine (R) 1496 and lysines (K) 1463 and

1464 are specifically involved. *C. elegans* worms in which these residues were

mutated to alanines or the entire PH domain of KIF1A was deleted mimicked the

Unc104 deletion phenotype (Klopfenstein and Vale, 2004). Specifically, these

mutant worms had an accumulation of SVPs in the cell body, a decrease in

synaptic vesicles at presynaptic sites and paralysis. PH domains interact with a

large variety of proteins in addition to lipids so the KIF1A PH domain mutants

could not be rescued by PH domains from other proteins (Klopfenstein and Vale,

2004). It is likely then that the PH domain of KIF1A interacts with specific vesicle

associated cargo in addition to the vesicular membrane. As is the case with

KIF5, KIF1A can bind cargo directly (SVPs) or through adaptor proteins (liprin-α and GRIP1). Of particular interest is whether these adaptor proteins play a role in the commonly observed bidirectional movement of axonal cargo, a phenomenon that involves kinesins and dyneins. The cargo linkers described here for kinesin, have not yet been implicated in axonal retrograde transport and in fact only one dynein associated cargo linker, dynactin, has also been shown to associate with kinesin (Colin et al., 2008).

1.9.4 Dynactin and Bidirectional Transport

Dynactin is a multiprotein complex known as a dynein-cargo linker. It anchors dynein to microtubules and facilitates dynein based cargo transport by driving the long-range processive movement of this molecular motor (Schroer, 2004). Mutations in dynactin cause neurodegenerative diseases that affect motor neuron function such as in amyotrophic lateral sclerosis (ALS) (Stokin and Goldstein, 2006) and Perry Syndrome (Farrer et al., 2009). These disorders are in part due to the failure of the mutant dynactin to facilitate the transport of cargo resulting in the breakdown of signalling pathways, eventually leading to neuronal degeneration. Recent evidence suggests that dynactin may also play a role in coordinating both dynein and kinesin motors in the bidirectional axonal transport of cargo (Schroer, 2004). Little is known about dynactins interaction with dynein and even less is known about its interaction with kinesin. There may be certain amino acid sequences which facilitate motor interaction with dynactin or alternatively three dimensional structures may be the basis of the interaction. It is not clear at this time how dynactin coordinates motor activity but what is clear

is that coordination of both kinesin and dynein is integral to proper axonal transport, cellular polarization and cellular function.

Dynactin consists of several subunits which come together to form a lever-like structure. There are two main structural features of the adaptor. The rod portion of dynactin contains an octomeric polymer of the actin related protein Arp1. The Arp1 rod is flanked by Arp11, p25, p27 and p62 on one end and the conventional actin capping protein CapZ on the other. The middle portion of the Arp1 rod associates with four copies of dynamitin (p50), two copies of p24/22 and two copies of p150 (Schroer 2004) (Fig. 3). The components of dynactin maintain the integrity of the dynactin structure and mediate its interaction with cargos, motors and adaptors.

The arm portion of dynactin consists of a single protein called p150^{Glued} which is by far the best studied dynactin component. Its N-terminal 110 amino acids contain globular cytoskeleton associated protein, glycine rich (CAP-Gly) motifs which bind microtubules and enhance motor processivity or participate in anchoring the motor to microtubules (Schroer, 2004). These domains also bind microtubule binding proteins such as CLIP-170 or EB1 which can regulate p150^{Glued} binding to the ends of microtubules (Magnani et al., 2007). The middle portion of p150^{Glued}, encompassing the first coiled coil region, interacts with dynein and kinesin. Dynein binding to this region of p150^{Glued} is often mediated by dynein intermediate chains (DIC) (King et al., 2003). Although it is known that the N-terminus coiled coil region of DICs binds somewhere between amino acid 600 and 811 of p150^{Glued} the exact amino acids involved in this interaction have

yet to be determined. This same region of p150 also binds the kinesin motors

Eg5 and Kinesin-2 (Berezuk and Schroer 2007). Thus both families of motors

bind to the same region of p150^{Glued} but the method by which this binding is

regulated is only beginning to be understood. The C-terminal region of p150^{Glued}

associates with the rod portion of dynactin and has also been shown to interact

with the microtubule associated protein tau. The N-terminal of tau binds to the C-

terminal of p150^{Glued} and facilitates its association with microtubules. In fact

certain mutations in the p150^{Glued} binding region of tau are associated with a

familial form of dementia called frontotemporal dementia and parkinsonism linked

to chromosome17 (FTDP-17) (Magnani et al., 2007). This neurodegenerative

disease may be a result of abnormal function of the dynactin complex and by

extension, impaired axonal transport.

Bidirectional movement of organelles along MTs has been observed in

many different organisms. It was hypothesised that this movement could occur

in several different ways. Individual plus or minus end motors could be recruited

to the vesicle at the time that they are needed. Alternatively both motors could

be bound to the cargo at the same time and engage in a tug of war to determine

the direction of travel or some sort of switching mechanism is used to activate

one of the motors in the direction of travel (Welte, 2004) (Fig. 4). The emerging

evidence seems to point to the latter hypothesis. In the late 90's it was

demonstrated that kinesin and dynein co-purify with melanosomes (Rogers et al.,

1997). Subsequent studies have reported observations of fluorescently labelled

organelles such as DCVs moving in both directions in live vertebrate axons

(Kwinter et al., 2009) as well as in invertebrate axons (mitochondria) (Pilling et al., 2006). Finally, when either the plus or minus end motor is disabled, transport is diminished in both directions indicating that both plus and minus end motors are bound to the same vesicle and that their activation is coordinated (Welte, 2004). Dynactin emerged as a prinicipal candidate for this motor coordination since overexpression of dynactin components such as portions of p150^{Glued} and dynamitin also decreased transport in both directions (Welte, 2004). Because the binding sites for DIC and kinesin overlap on p150^{Glued} it may be that dynein and kinesin cannot bind p150^{Glued} at the same time and that one of the motors (perhaps the one not in use) is tethered to the same vesicle by some other means. In Xenopus melanophores, melanosomes aggregate and disperse due to the co-ordinated work of dynein and kinesin-2. Both motors associate with dynactin on these organelles through the accessory proteins DIC and KAP respectively but they do not associate with each other. KAP and DIC bind the same area of p150^{Glued} (amino acids 600-811) but are unable to do so at the same time (Deacon et al., 2003). In mammalian cortical neurons the phosphorylation of huntingtin, a protein known to associate with DICs, recruits kinesin-1 heavy chain (KHC) to dynactin and drastically enhances the interaction between p150^{Glued} and kinesin (Colin et al., 2008). Perhaps then, when a motor is not in use it is bound to other portions of dynactin and its binding to p150^{Glued} can be regulated through linker proteins. Thus, it may be that motor binding to p150^{Glued} releases the motor from some sort of inhibitory state and determines vesicle directionality. Only individual members of the kinesin-1, kinesin-2 and

kinesin-5 families (KHC, KIF3A/3B and Eg5 respectively) have been shown to bind dynactin (Schroer, 2004; Uteng et al., 2008). The binding of other kinesins to dynactin has not yet been explored. Some evidence suggests that KIF1A also binds dynactin as dynactin is known to interact with both SVPs and DCVs (Koushika et al., 2004; Park et al., 2008; Kwinter et al., 2009). The previously mentioned study by Park et al., (2008) co-immunoprecipitated KIF1A with p150^{Glued} out of mouse brain cytosol where both proteins are associated with carboxypeptidase E (CPE) and BDNF containing DCVs (Park et al., 2008). Thus, evidence suggesting that bidirectional transport is achieved through the coordinated action of both plus and minus end motors is mounting. This coordination seems to involve the p150^{Glued} arm of dynactin and several plus end motors have already been found to bind p150 in addition to dynein. However, the method by which motors bind dynactin and how dynactin regulates their co-ordinated movement is yet poorly understood. Specifically, it is not clear what role, if any, other linker proteins play in co-ordinating bidirectional transport and how this may involve dynactin. One study identified the protein lipid storage droplet protein 2 (LSD2) as a potential regulator of bidirectional lipid droplet distribution in *Drosophila* embryos (Welte et al., 2005). Their experiments indicate that LSD2 phosphorylation, similarly to Htt phosphorylation, causes a directed change in the direction of lipid droplet transport (Welte et al., 2005). Unfortunately the authors of this paper did not investigate the role of dynactin in lipid transport so it is not clear if is needed or not. How bidirectional transport is regulated would specifically shed light on axonal transport where the polarity of

microtubules imposes the use of both plus and minus end motors not only to deliver cargos to and from the cell body put to navigate the densely packed environment of the axon. DCVs are no exception to bidirectional axonal transport. This thesis helps to define mechanisms of plus end DCV movement and also contributes to the understanding of bidirectional DCV axonal transport.
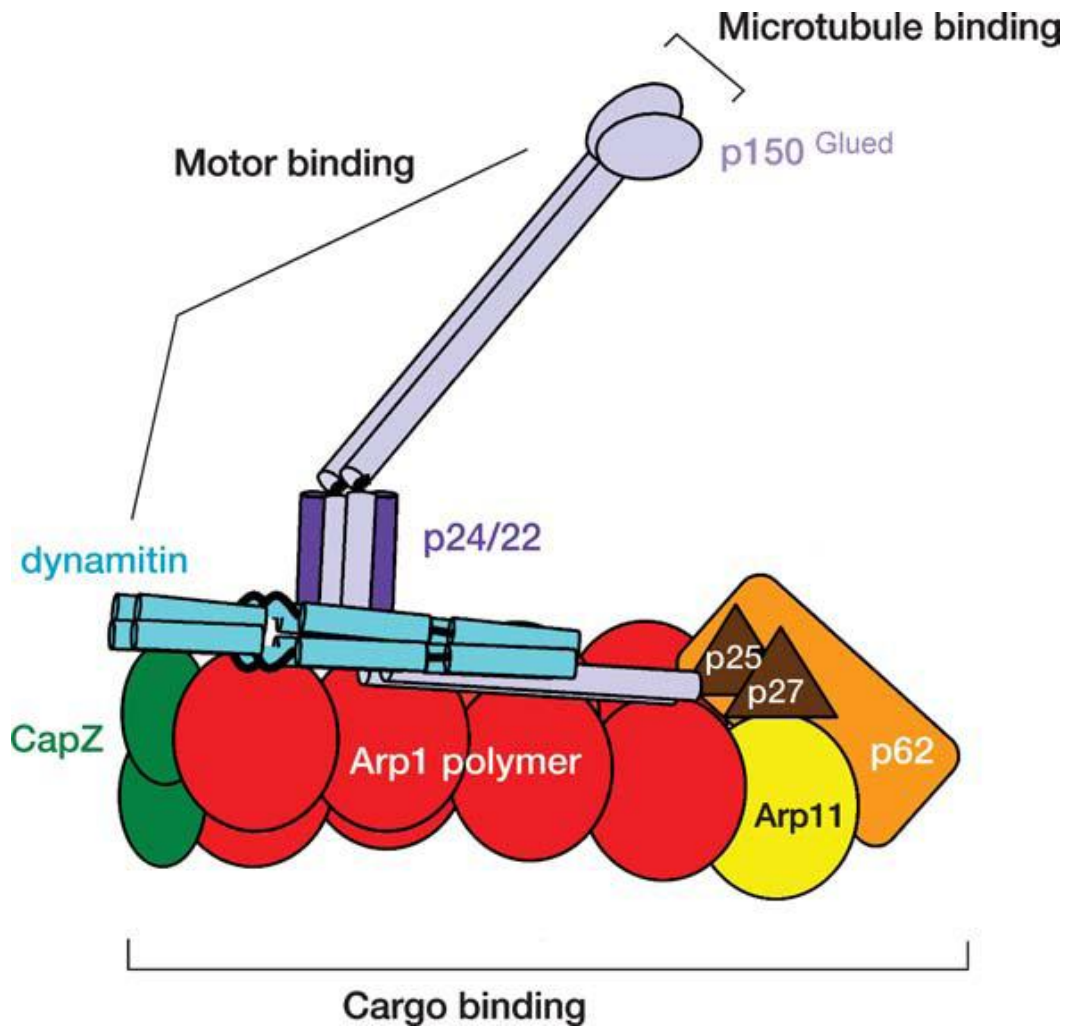
Figure 3. Structure of Dynactin

The structure of dynactin showing the microtubule, motor and cargo binding portions. *Schroer, 2004*
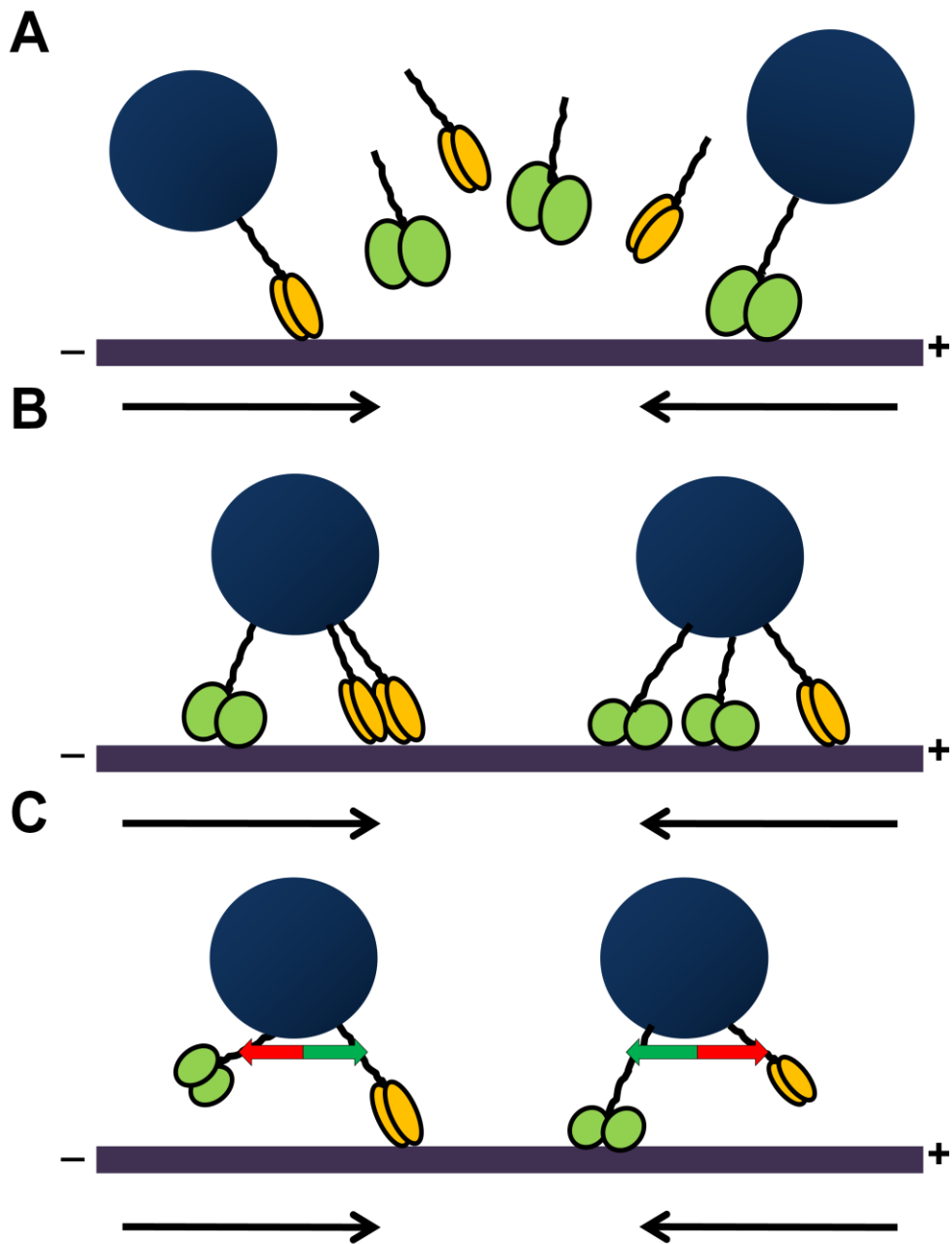
Figure 4. Three Models Used to Describe Bidirectional Transport

A, Plus-end (yellow) or minus-end (green) motors bind cargo individually and each is recruited from the cytoplasm based on need. *B*, Several plus or minus-end motors could be attached to the cargo and active at the same time, engaging in a tug of war where the greater number of plus or minus end motors determines transport directionality. *C*, Both motors could be attached to cargo at the same time but only one motor is active at any given moment because some kind of switching mechanism (red and green double-headed arrow) determines which motor is in use. *(*Adapted from *Welte, 2004)*

1.10 Live Imaging and Cultured Hippocampal Neurons

The ability to visualise the movement of single vesicles or particles required a different approach due to the limitations in the resolution of such small particles by conventional light microscopes. This approach came with the relatively recent development of fluorescent protein coupled dyes and genetically encoded fluorescent proteins, which have allowed scientists to track the movement of single particles in neurons using wide field microscopy. The improvement of the charged coupled device (CCD) camera sensitivity in the 90s permitted the tracking of single fluorophores in living cells (Triller and Choquet, 2008). Such technologies underlie many studies (including this one) of axonal transport in cultured living neurons and are widely used in the field of neurobiology.

Hippocampal neurons are one of the primary accepted model systems in which to study neuronal polarity and trafficking and so are used exclusively in this study. Hippocampal neurons in culture closely resemble those in vivo in form and function (Goslin and Banker, 1989). This is supported by examples such as the dendritic microtubule associated protein, MAP2 and axonal GAP-43 growth-associated protein that will localize in cultured hippocampal neurons as they would in vivo (Craig and Banker, 1994). This study used two fluorescent proteins, green fluorescent protein (GFP) and red fluorescent protein (RFP), to track the movement of DCV associated proteins in cultured living hippocampal neurons using wide field microscopy. The purpose was to determine the role of KIF1A in DCV transport in mammalian neurons based on the discovery of KIF1A

dependent DCV transport in invertebrates. Firstly, RNAi against endogenous KIF1A was used to determine its role in axonal DCV transport. Secondly, the movement of fluorescently labelled KIF1A and BDNF were tracked by near-simultaneous two-colour imaging in live cultured rat hippocampus neurons. With these techniques, this thesis identifies KIF1A as the primary motor responsible for the axonal transport of DCVs in vertebrates and provides functional evidence for the bidirectional axonal movement of KIF1A. The work is important for understanding cargo delivery with respect to proper transport fidelity and overall neuronal function.

2: METHODS

2.1 Hippocampal Cell Culture and Expression of Transgenes

Primary cultures of dissociated neurons from rat embryonic day 18 (E18) hippocampi were prepared essentially as described (Kaech and Banker, 2006). The dissociated neurons are plated onto poly-L-lysine (Sigma-Aldrich) pre-treated glass coverslips and then the coverslips are placed over a monolayer of astrocytes with the neurons facing down. Constructs were transfected into neurons after 8 days *in vitro* (DIV) and allowed to express for 24-48 hours before imaging. Immediately prior to transfection neurons were treated with 0.5 μM kynurenic acid (Sigma-Aldrich) to decrease excitotoxic damage. Lipofectamine 2000 (Invitrogen) was used to transfect cells with 1 μg of each plasmid, according to manufacturer's instructions as described by Sampo et al., (2003). All cultures were maintained at 37 ºC under a controlled atmosphere containing 5% CO_2. In preparation for Western blot analysis of KIF1A expression in control and knockdown cells, E18 hippocampal neurons were electroporated with constructs using the AMAXA nucleofector II electroporator according to manufacturer's instructions and Zeitelhofer et al., (2007). Cells were electroprated immediately after dissection and allowed to culture for 48 hours. Prior to protein extraction one coverslip of neurons from each condition was assessed for transfection rate by counting the proportion of GFP expressing cells. Proteins were extracted on ice, by scraping cells from the coverlips (4

cover slips per sample) in 200 µl of phosphate buffered saline (PBS). Samples were then sonicated briefly to further break up the cells and then heated to 100 ºC for 5 min. Laemmli buffer was then added to each sample and the samples were stored at -80 ºC.

2.2 Constructs

An siRNAi sequence directed against the same 21 base pair region within the forkhead association (FHA) domain (amino acids 527-571 as defined by SMART) of rat and mouse KIF1A mRNA was chosen based the demonstration of its efficacy by Dr. Gary Banker, Oregon Health Sciences University, USA. The rat KIF1A RNAi sequence encompassed base pairs (bp) 1689-1709 (5'TACCTAT GTGAACGGCAAGAA-3') as defined by Ensembl (ENSRNOG00000023993) based on Genbank entry XM_001070204. The mouse KIF1A mRNAi encompassed bps 1842-1862 (5'CACATATGTCAACGGCAAGAA-3') as defined by Ensembl (ENSMUSG00000014602) based on Genbank entry NM_008440. To construct the hairpin RNA structure for both constructs the forward RNAi sequences were followed by the reverse complement separated by a short linker sequence that contains an EcoRV restriction site. To facilitate cloning an HpaI was incorporated at the 5' end and a Xho I site was incorporated at the 3' end of both constructs. The sequence 5'**AAC**TACCTATGTGAACGGCAAGAAT T**GATATC**CG<u>TTCTTGCCGTTCACATAGGTA</u>**TTTC**-3' containing the rat KIF1A RNAi and the sequence 5'**AAC**<u>CACATATGTCAACGGCAAGAA</u>TT**GATATC**CG

<u>TTCTTGCCGTTGACATATGTG</u>**TTTC**-3' containing the mouse KIF1A RNAi

(RNAi forward and reverse sequences are underlined, restriction sites in bold)

and their respective complements which contain a 5' phosphate at the Xho I

restriction site, were ordered from Integrated DNA Technologies (IDT). The

complementary strands were annealed in a Tris-HC, 1M NaCl, annealing buffer

by gradual cooling from 95 ºC.

The annealed double stranded sequences were cloned into a pLentilox-

GFP (pLox) vector, kindly sent by Dr. Gary Banker, Oregon Health Sciences

University, USA, using standard cloning techniques. The annealed fragments

were cloned next to the CMV-U6 promoter using the restriction sites HpaI and

XhoI. This site is located upstream of the GFP sequence expressed from a CMV

promoter. The GW1 KIF1A-GFP plasmid contains GFP cloned between Ser-680

and Arg-681 of KIF1A, as described in Lee et al., 2003). Fluorescent NPY-

mCherry was constructed as described in (Lochner et al., 2008; Silverman et al.,

2005). N-terminal myc-tagged, RNAi resistant KIF1A, BDNF-RFP, and TfR-GFP

were donated by Dr. Gary Banker, Oregon Health Sciences University, USA.

The mitochondrially targeted enhanced yellow fluorescence protein (eYFP)

expression vector DNA was a kind donation from Dr. Gordon Rintoul, Simon

Fraser University, Canada.

2.3 Immunoblotting and Immunocytochemistry

Proteins were extracted from electroporated cells as described above,

mixed with Laemmli buffer and 5 µg of each sample was run on a 10% SDS

polacrylamide gel. The gel was probed with monoclonal anti-KIF1A (BD

Biosciences) 1:500 and monoclonal anti-α-tubulin (Sigma-Aldrich) (1:2000).

Blocking, prior to antibody addition, was performed in 1% BSA. Bands were

visualized with goat anti-mouse-HRP (BioRad) secondary antibody (1:30,000)

and SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Protein

Research Products) on autoradiography film (Midsci™). Relative protein

amounts were quantified by analyzing band intensity with ImageJ. Cells rescued

with myc-RNAi resistant KIF1A were fixed in 4% paraformaldahyde/ 4% glucose

and permeabilized in 0.1% Triton X-100. Cells were blocked for 1hr at 37 ºC in

fish skin gelatin and BSA (0.5%, 10%, Sigma-Aldrich). Primary and secondary

antibodies were also diluted in fish skin gelatin and BSA. After blocking the cells

were probed with polyclonal anti-myc (Sigma-Aldrich) primary antibody (1: 280)

overnight at 4 ºC. Cells were then washed with PBS and subsequently incubated

with donkey anti rabbit Cy5 (Jackson Immunoresearch Labs) secondary antibody

(1:500) for 1hr at 37 ºC.

2.4 Microscopy and Live Imaging

Cells were imaged live using a standard wide-field fluorescent microscope (DMI

6000 B, Leica Microsystems AG, Wetzlar, Germany) which is equipped with an

Orca cooled CCD camera (Hamamatsu Photonics KK, Bridgewater, NJ, USA).

The camera is controlled by *MetaMorph* Software (Universal Imaging,

Downingtown, PA, USA). During imaging cells resided in a sealed 37 °C

temperature-controlled chamber (Warner Instruments, Hamden, CT, USA) which

contained pre-warmed imaging medium (1 X Hanks' with Ca^{2+} and Mg^{2+}, 0.6%

glucose, and 10 mM HEPES). All movement which was recorded by sequential

250 ms (streaming) exposures for 200 frames (50 s) or by two colour imaging

was obtained with a 63X 1.4 NA plan apochromat temperature-controlled

objective set at 37 °C. Additionally, still images of cells were obtained with a 20X

1.4 NA objective. During two-colour imaging 30 exposures of 250 ms were taken

alternately with GFP and texas red filters. There was a 0.5 second delay

between the filter switching.

2.5 Data Analysis

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). DCV flux was

measured as the distance of DCV run lengths in µm standardized by the length

of the axon imaged and duration of the filming (µm x min): $\sum_{i=1}^{n} d_i / (l \times t)$ where d

is the individual DCV run lengths (µm), *l* is the length of axon observed (µm) and

t is the duration of filming (50 seconds). Vesicles travelling 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 $\sqrt{2Dt}$

where D is the diffusion coefficient (D = 0.01 $\mu m^2/s$ for DCVs) 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). 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 Microsoft Excel.

3: RESULTS

## 3.1 KIF1A and BDNF co-localize in living cultured hippocampal neurons

Definitive evidence of protein co-transport is derived from tracking the movements of each type of molecule in living cells. If the location and movements of two labeled proteins are identical, that is, they start together, travel at the same rates, and stop together, it is likely they reside on or in the same organelle. To determine if KIF1A associates with DCVs I co-expressed KIF1A-GFP and BDNF-RFP in primary cultured hippocampal neurons and examined the cells by two-colour live-cell imaging (Fig.5 and Fig. 6). I evaluated the degree of co-transport in the axon by comparing pairs of kymographs generated for each colour-tagged protein (Fig. 7; n=16 movies from 13 cells). The percentage of KIF1A co-transporting with BDNF was 70% ± 11.2% in the anterograde direction and 64% ± 17.5% in the retrograde direction. The percentage of BDNF co-transporting with KIF1A was 47% ± 10.6% in the anterograde direction and 40% ± 10.3% in the retrograde direction (Fig. 8). These results indicate that in the axon not only do KIF1A and DCVs move in tandem but that they do so almost equally in both the anterograde and retrograde direction.

43
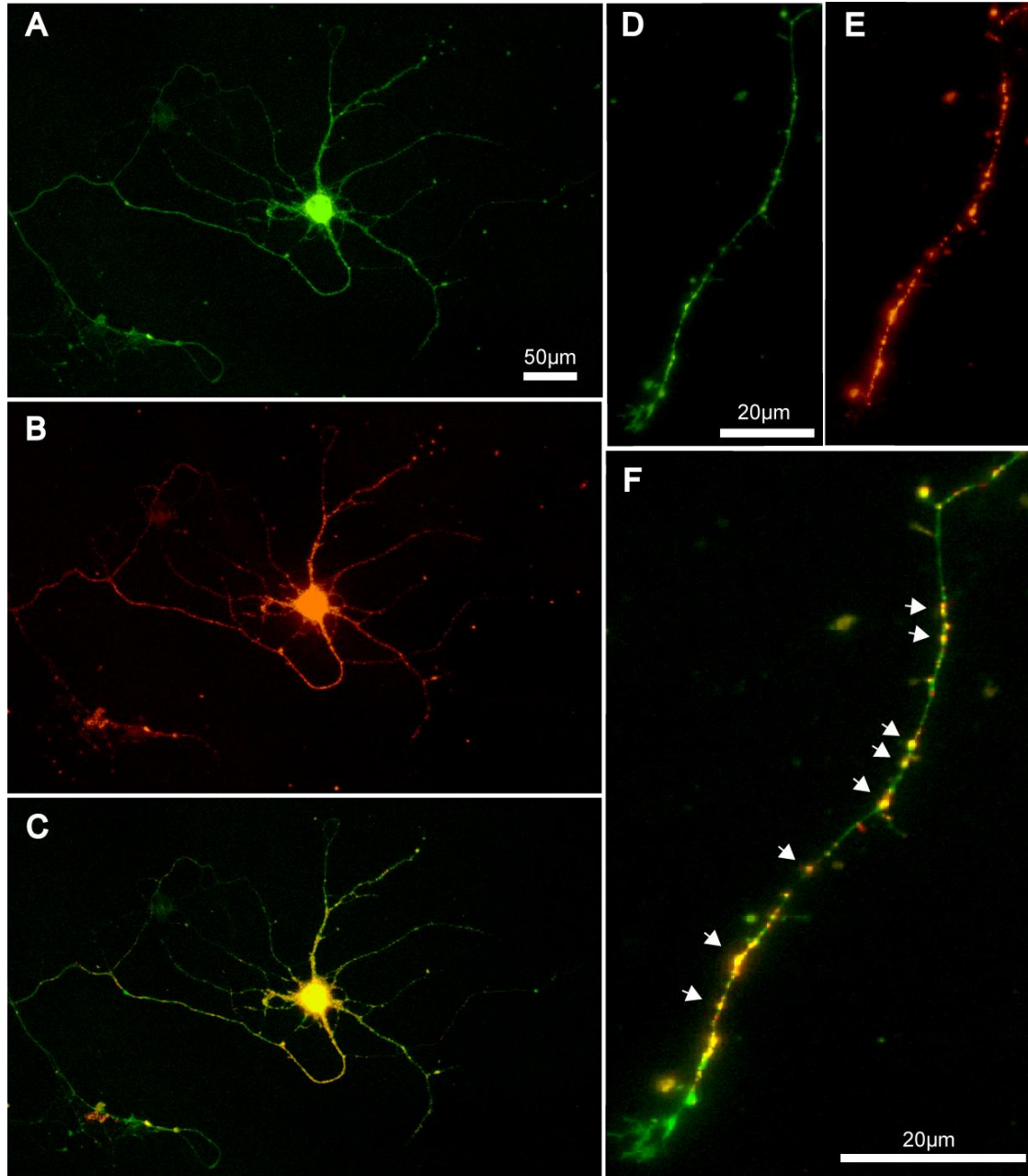
Figure 5. Expression of KIF1A-GFP and BDNF-RFP in Rat Hippocampal Axon

Cultured live hippocampal neuron expressing KIF1A-GFP and BDNF-RFP. *A*, Low magnification (200x) of a neuron expressing KIF1A-GFP, *B*, BDNF-RFP. *C*, Overlay of (A) and (B). Distinct puncta of KIF1A-GFP (D) and BDNF-RFP (E) are visible within an axon under higher magnification (630x). *F*, Overlay of (D) and (E) reveals many areas of co-localization (arrows pointing to yellow spots) between the two markers.
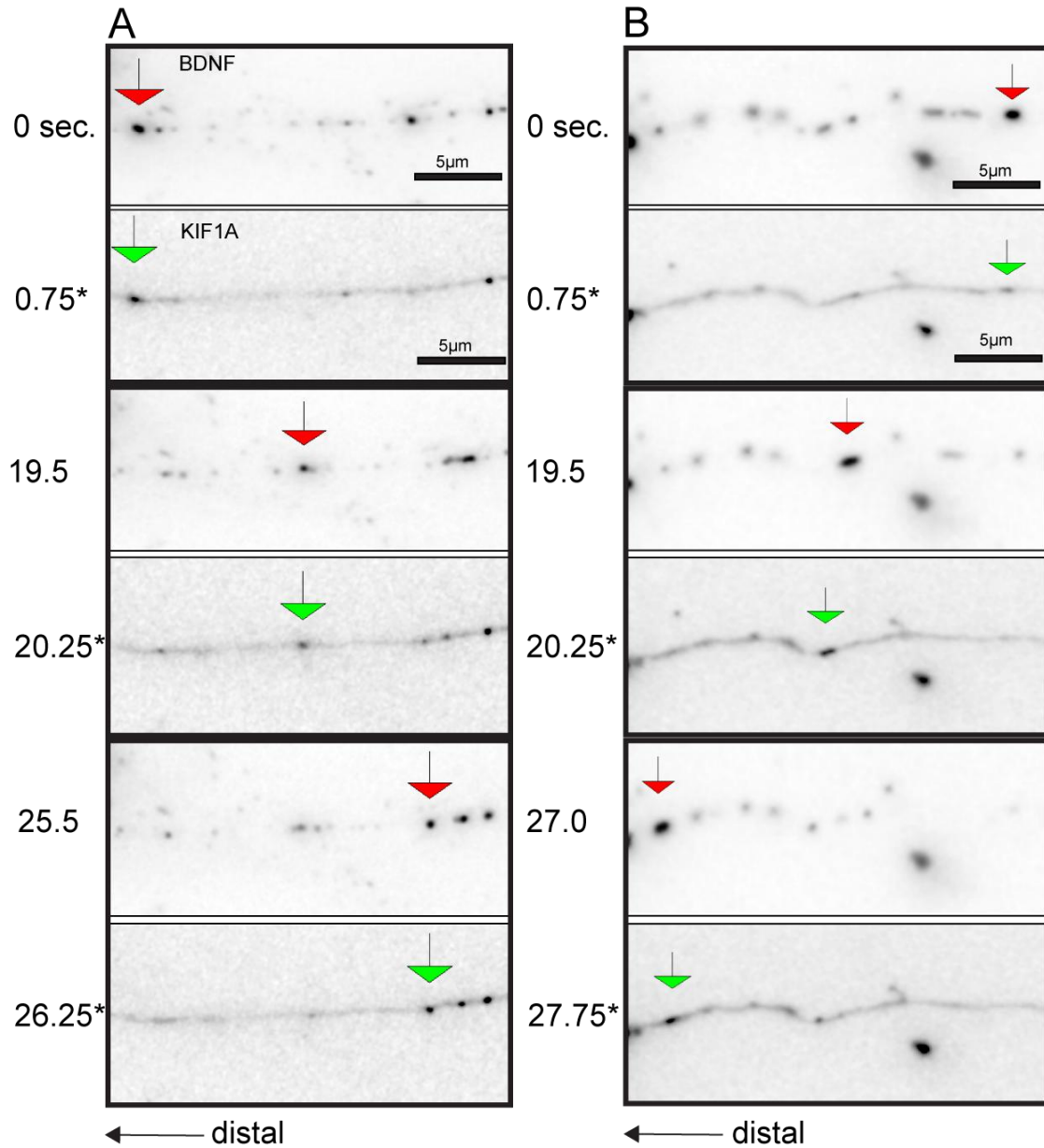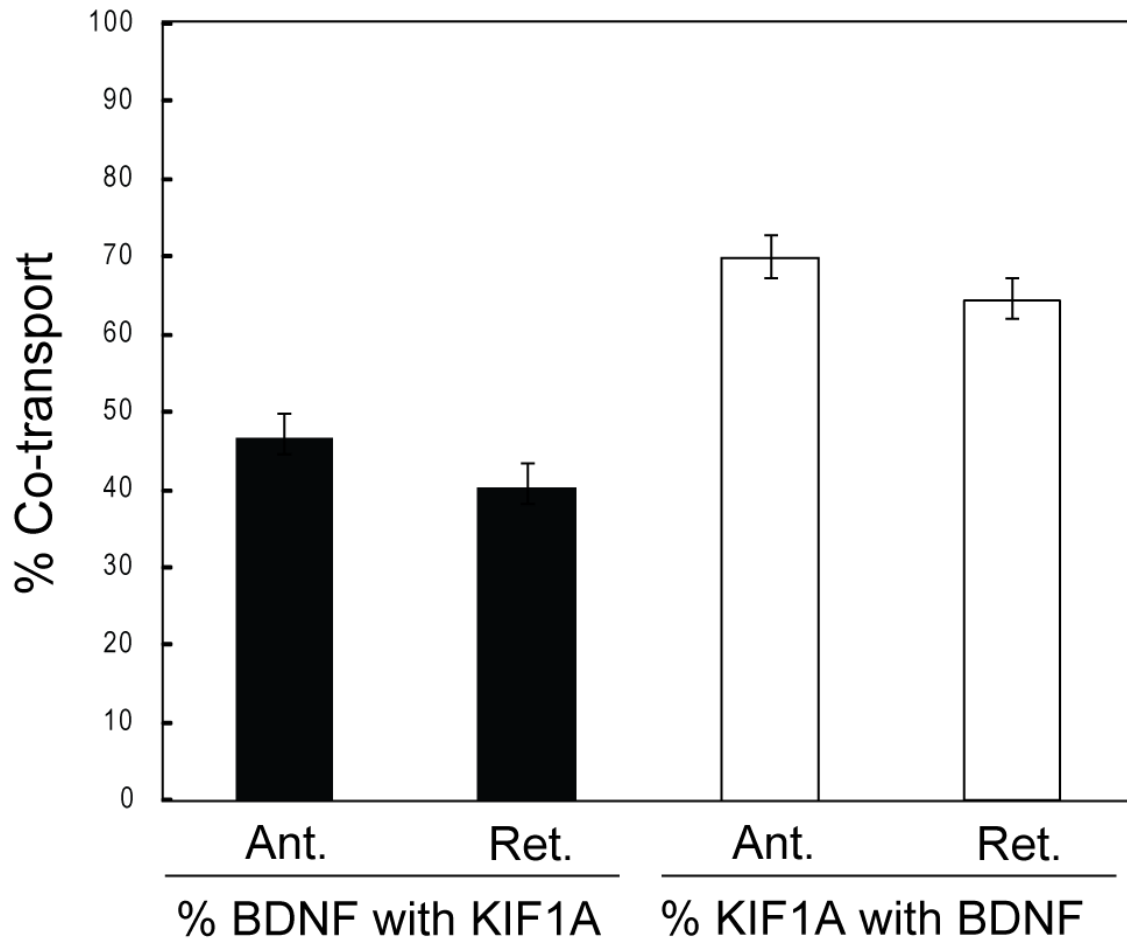
Figure 6. KIF1A-GFP and BDNF-RFP Move Together in Both Directions

Representative movie frames showing the movement of KIF1A-GFP and BDNF-RFP in living hippocampal axons (boxed in frames). Movies were made at a magnification of 630x using two-colour imaging and were a total of 45 seconds long. There is a 0.5 second delay between frames of different colour to account for the time it takes to switch between filters. Each exposure is 250ms. Panels are shown in monochrome for clarity. Time in seconds is shown on the left. Times followed by an asterisk designate the KIF1A frame (green arrows) taken subsequent to the preceding BDNF frame (red arrows, no asterix). *A*, Retrograde cotransport of BDNF and KIF1A (average velocity 0.76µm/s). *B*, Anterograde co-transport of BDNF and KIF1A (average velocity 0.72 µm/s).
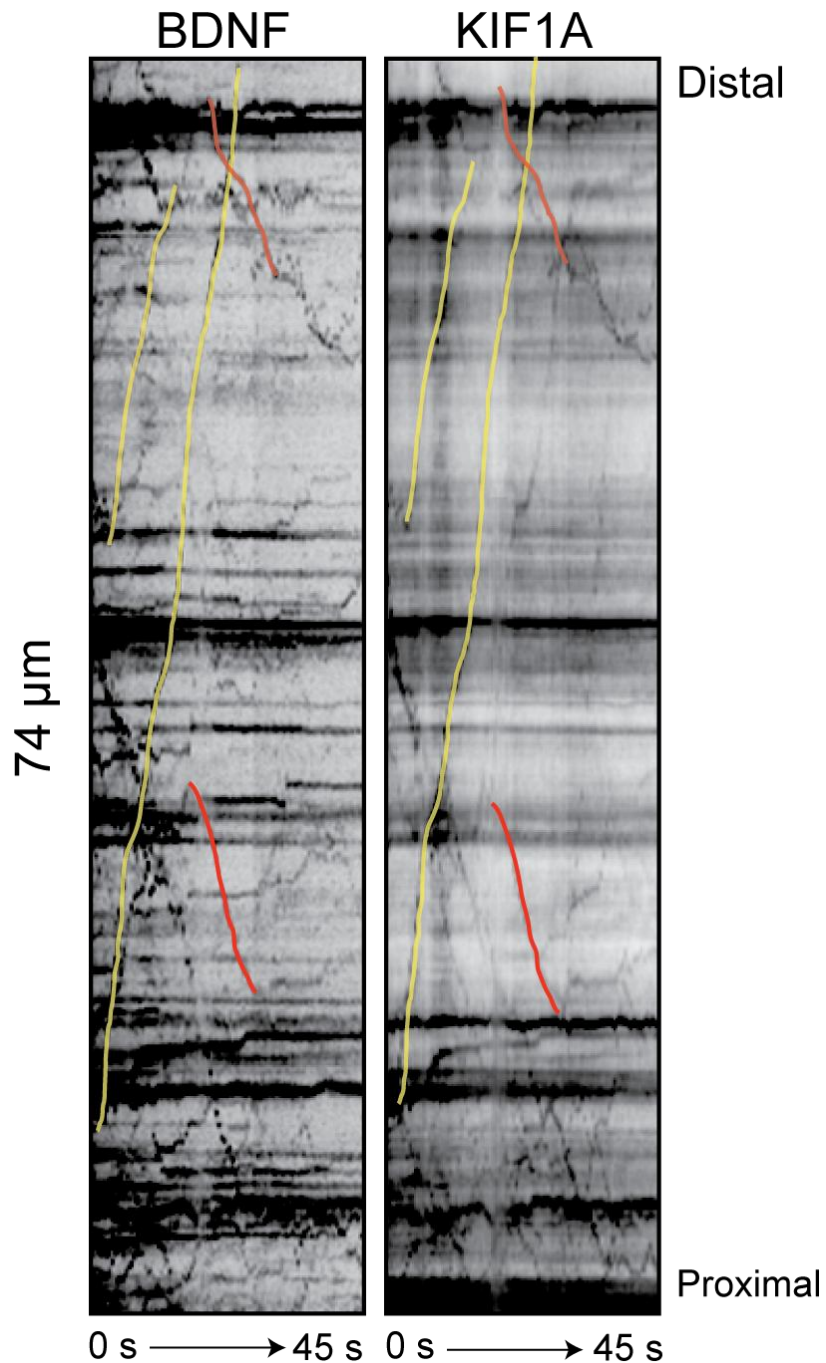
Figure 7. Kymographs Generated From KIF1A-GFP and BDNF-RFP Movies

Kymographs show movement of fluorescently labelled particles through time within a portion of a single axon. Time is represented on the X-axis and the length of axon analyzed is on the Y-axis. Yellow lines show examples of particles moving in tandem anterogradely and red lines are examples of particles moving in tandem retrogradely. Horizontal lines are stationary particles. To determine the degree to which lines co-localize, all lines were traced on each kymograph individually and then superimposed. The overlapping lines were manually counted to determine the percentage of co-localization of either KIF1A with BDNF or BDNF with KIF1A.

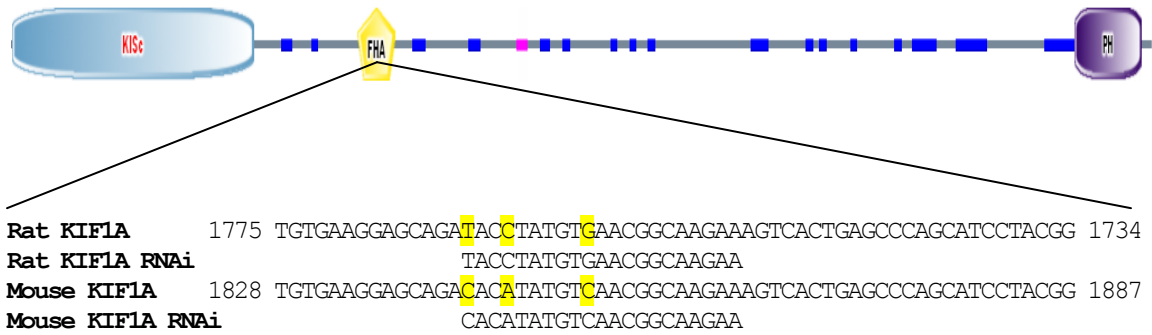**Figure 8. KIF1A and BDNF Move Together in Axons**

The amount of co-transport between KIF1A-GFP and BDNF-RFP was analyzed by superimposing diagonal lines traced on the kymographs generated from each movie. The dark bars show the percentage of BDNF traces that co-localized with KIF1A traces and the light bars show the percentage of KIF1A traces that co-localized with BDNF traces. Ant.=Anterograde, Ret.= Retrogade

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## 3.2 Rat KIF1A RNAi Decreases the Amount of DCV Movemement

3.2.1 Rat KIF1A RNAi Knocks Down Endogenous KIF1A

To determine if KIF1A has a functional role in DCV transport I used siRNA designed against a 21 base pair region within the forkhead (HA) domain of endogenous rat KIF1A mRNA. To control for off-target effects of the rat KIF1A RNAi, I also used RNAi against the same region of mouse KIF1A mRNA (Fig. 9). In order to identify transfected cells I inserted the siRNA into an expression vector that also expresses soluble GFP. Neurons were electorporated with control (mock and mouse KIF1A RNAi) and knockdown plasmids. I evaluated the transfection efficiencies of each electroporation by counting the percentage of fluorescence emitting cells which was 47% for mouse KIF1A RNAi and 41% ± 0.07% for rat KIF1A RNAi. After 48hrs in culture I extracted proteins from the transfected cells in order to assess the efficacy of the rat KIF1A RNAi by immunoblot. The ratio of the intensity of KIF1A and tubulin bands in control cells was 1.4 ± 0.06 whereas the ratio of the intensity of these bands in knockdown cells was significantly lower, 0.2 ± 0.03 ($p < 0.01$). In knockdown cells there was a decrease of 86.74% in the intensity of the KIF1A band with respect to the tubulin band, indicating a decrease in endogenous KIF1A (Fig. 10 A, B).

Rat KIF1A   1775 TGTGAAGGAGCAGA`TACC`TATGT`G`AACGGCAAGAAAGTCACTGAGCCCAGCATCCTACGG 1734
Rat KIF1A RNAi   TACCTATGTGAACGGCAAGAA
Mouse KIF1A   1828 TGTGAAGGAGCAGA`CACA`TATGT`C`AACGGCAAGAAAGTCACTGAGCCCAGCATCCTACGG 1887
Mouse KIF1A RNAi   CACATATGTCAACGGCAAGAA

Figure 9. RNAi Directed Against KIF1A Forkhead Domain

RNAi was designed against a 21 base pair region within the KIF1A forkhead domain. 60 base pairs surrounding the targeted region of rat and mouse KIF1A are shown along with the RNAi sequences designed for each region. The three base pair differences between rat and mouse sequences within the RNAi region are highlighted in yellow.
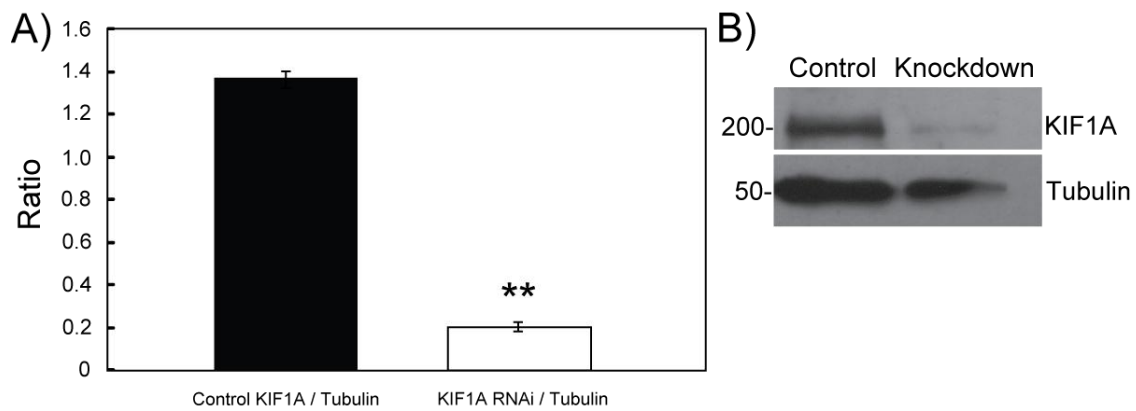
Figure 10. RNAi Against Rat KIF1A Knocks Down Endogenous Protein

A, The ratio of the intensity of KIF1A and tubulin bands from the western blots of two separate transfections of cells with control (n=2) and knockdown (n=2) plasmids. The ratios of KIF1A to tubulin obtained for each treatment were averaged between the two replicates. *B*, Immunoblot showing the endogenous KIF1A levels in control and experimental cells. Cell lysates were run on a 10% SDS-PAGE gel and analyzed by western blot with monoclonal anti-KIF1A (1:500) and monoclonal anti-tubulin (1:3000) antibodies. ** $P < 0.01$.

3.2.2 DCV Flux is Significantly Reduced in Rat KIF1A RNAi Expressing Cells

DCV movement was filmed in control and knockdown cells for a total of 50 seconds. Kymographs generated from cells in which rat KIF1A was knocked down had significantly fewer anterograde and retrograde DCV events (Fig. 11). DCV flux was measured as the distance of DCV run lengths in μm standardized by the length of the axon imaged and duration of the filming (μm x min): $\sum_{i=1}^{n} d_i$ / (l x t) where d is the individual DCV run lengths (μm), l is the length of axon observed (μm) and t is the duration of filming (50 seconds). Vesicles travelling less than 2 μm were not included in the analysis as such a short distance could be achieved by diffusion as described in Kwinter et al., (2009). This measurement of DCV movement does not only quantify the moving fraction of DCVs but also describes the amount of movement observed by this fraction by including DCV run lengths in the equation (Kwinter et al., 2009).   Average DCV flux in KIF1A knockdown cells was 0.7 ± 0.15 min^{-1}, significantly less (p < 0.01) than in empty vector and mouse KIF1A RNAi where average DCV flux was 3.6 ± 0.9 min^{-1} and 3.0 ± 0.3 min^{-1} respectively (Fig. 12 and Table 2). No significant difference was found between DCV flux in empty vector treated cells and DCV flux in mouse RNAi expressing cells. Total flux includes both anterograde and retrograde movement and so it is important to note that both of these were significantly decreased in KIF1A knockdown cells. Anterograde and retrograde flux were also measured independently and each of these was also significantly decreased in experimental cells compared to controls (Table 2).

In addition to overall DCV flux, DCV velocity and run length were also measured. Average anterograde and retrograde DCV velocity in control cells (1.3 ± 0.04 µm/s and 1.4 ± 0.04 µm/s respectively) were not significantly different from the moving fraction in knockdown cells (1.25 ± 0.06 µm/s and 1.3 ± 0.06 µm/s respectively) (Fig. 13, Fig 15 and Table 2). Control and knockdown values for velocity agree with previously reported KIF1A velocities (Hirokawa and Noda, 2007), and dynein velocities (Roy et al., 2000) supporting the notion that DCVs are carried by KIF1A and possibly dynein.

Average anterograde DCV run length was not affected in the moving fraction of DCVs in knockdown cells (5.5 ± 0.7 and 6.2 ± 0.04 µm respectively) but retrograde run length was decreased (p < 0.05) in the moving fraction of knockdown cells compared to controls (5.1 ± 0.4 and 6.4 ± 0.6 µm) (Fig. 14, Fig. 15 and Table 2). Control and knockdown run lengths are within the range of values considered to be processive suggesting that the motor carrying DCVs is highly processive, as KIF1A is known to be (Okada and Hirokawa, 1999).
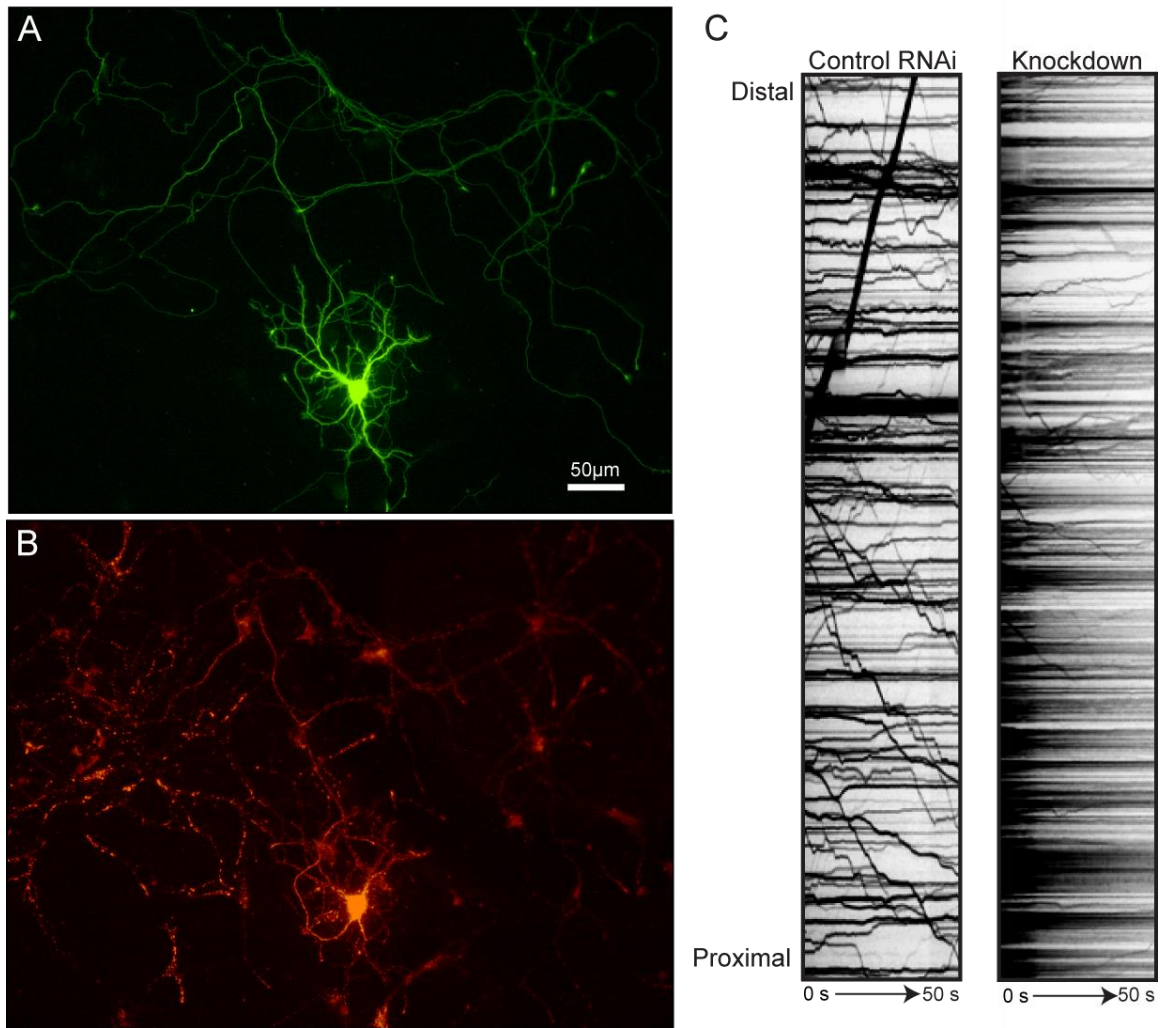
Figure 11. Kymographs Were Used to Analyze DCV Movement in Control and Experimental Cells

A, An example of a live neuron expressing soluble GFP indicating that it is transfected with the mouse KIF1A RNAi containing plasmid . *B*, The same neuron expressing the DCV marker BDNF-RFP. *C*, Kymographs were generated by *Metamorph* from DCV marker movement in control (left) and knockdown (right) cells. All lines on kymograph represent the movement of DCV marker BDNF over the course of 50 seconds. Therefore the movement of the particles through time is characterized by the diagonal lines.   DCV flux, velocity and run length were analysed based on the manual tracing of diagonal lines where 1 pixel = 0.160508 µm (y axis) and 4 pixels = 250ms (x axis).
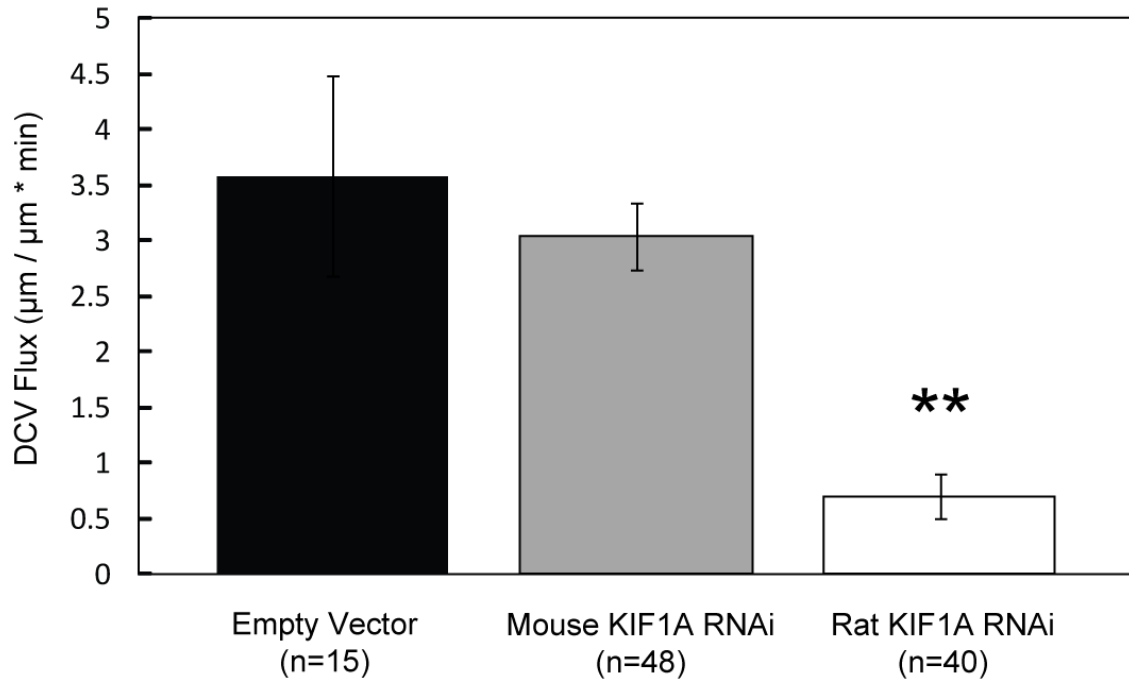
Figure 12. Knocking Down KIF1A Significantly Decreases DCV Flux.

Movies recording the movement of DCV markers BDNF and NPY were analyzed for overall DCV flux.   Average DCV flux in empty vector and mouse KIF1A RNAi controls are reported separately. Knockdown cells had a significant decrease in overall DCV flux compared to both sets of controls.   ** $P < 0.01$
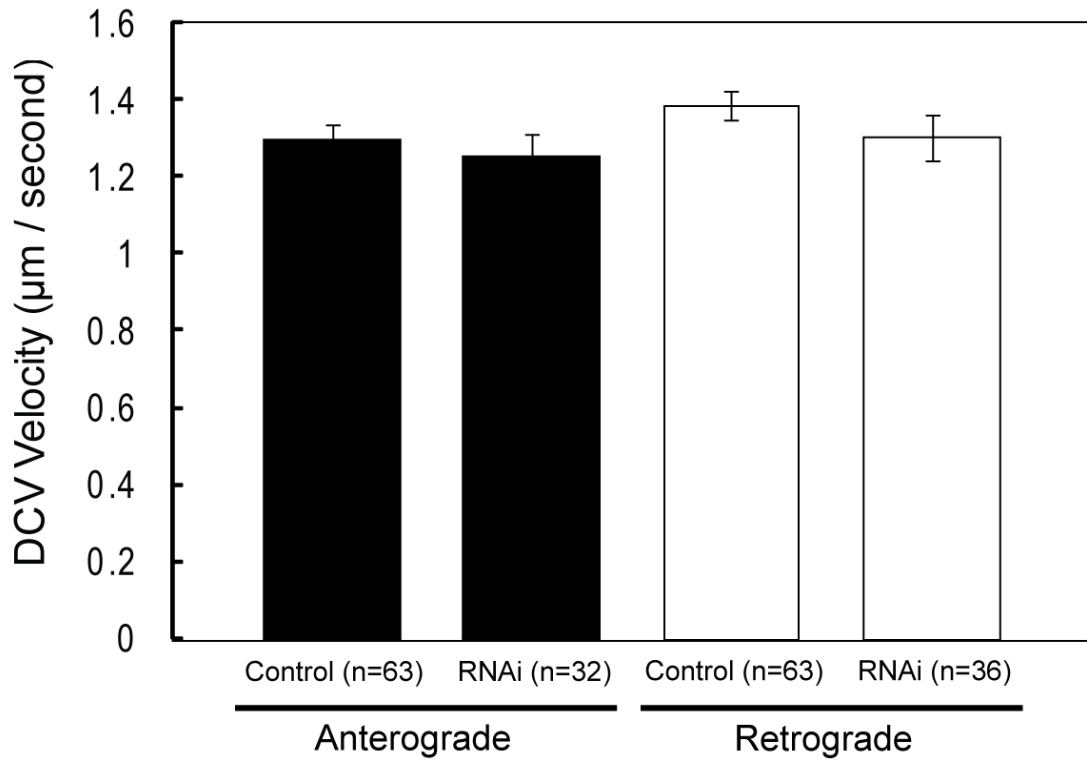
Figure 13. Anterograde and Retrograde Velocity in Control and Experimental Cells

Kymographs of control cells and experimental cells (RNAi) were analyzed for anterograde and retrograde velocity. Knockdown cells which had any zero values for anterograde or retrograde transport were removed from the analysis. The moving fraction in knockdown cells did not have a significant difference in anterograde or retrograde DCV velocity compared to control cells.
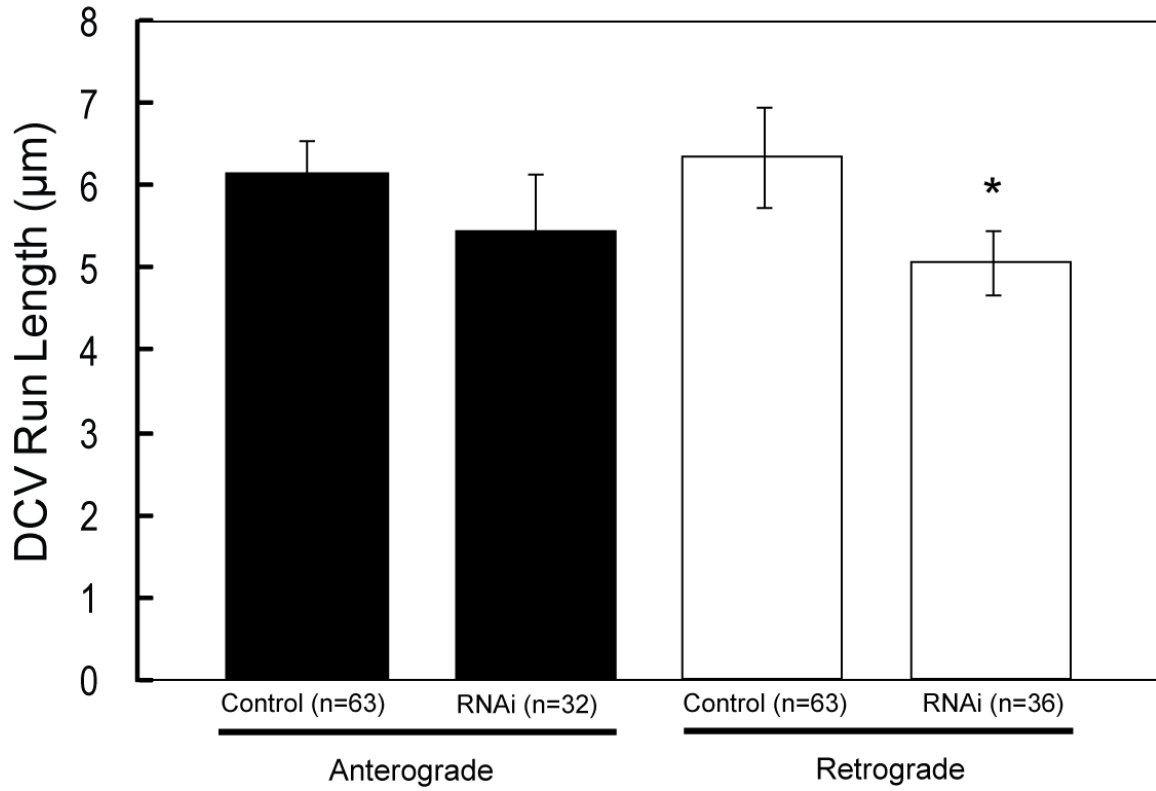
Figure 14. DCV Run Lengths in Control and Experimental Cells

Run lengths were measured as the total distance a given DCV marker travelled over a period of 50 seconds. Only the moving fraction in knockdown cells was analysed. Knockdown cells (RNAi) have decreased retrograde run lengths but not anterograde run lengths compared to controls. * $P < 0.05$.
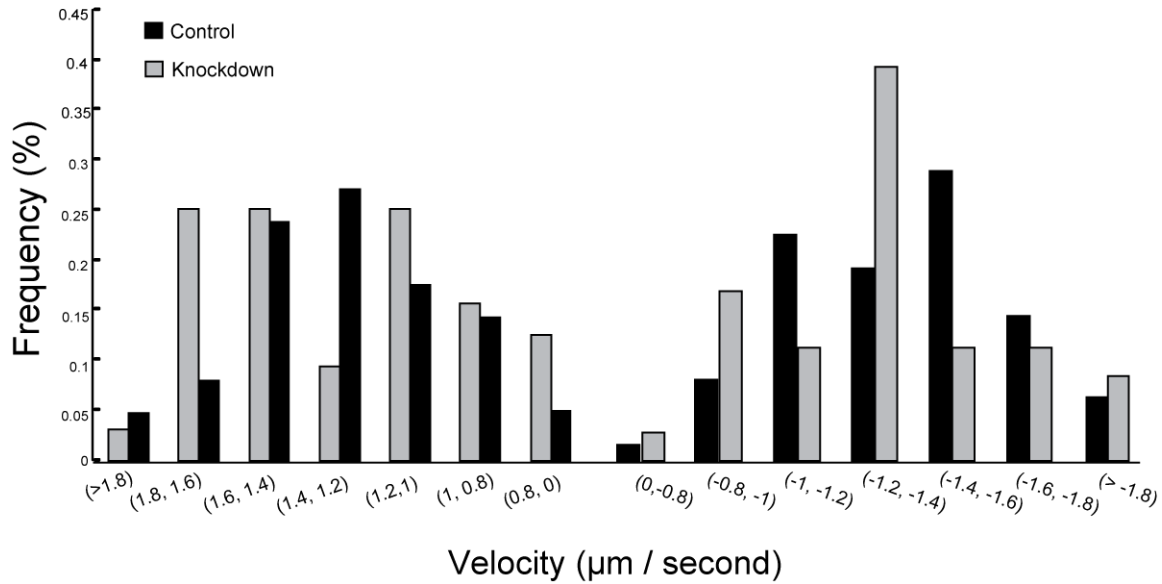
Figure 15. Velocity Distribution in Control and Knockdown Cells

Histograms showing the distribution of anterograde and retrograde DCV velocities in control (n=63) and knockdown cells (n=32 cells with anterograde movement; n=36 cells with retrograde movement). Anterograde velocity is shown on the left with positive numbers specifying the range of velocities. Retrograde velocities are shown on the right with negative numbers specifying the range of velocities.
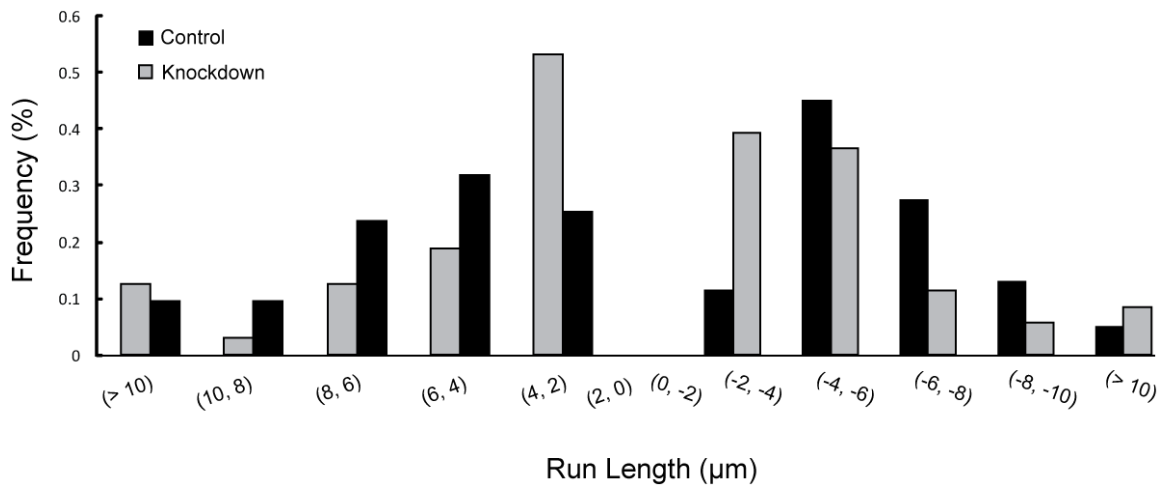
Figure 16. Run Length Distribution in Control and Knockdown Cells

Histograms showing the distribution of anterograde and retrograde run lengths in control (n=63) and knockdown cells (n=32 cells with anterograde movement; n=36 cells with retrograde movement). Anterograde run lengths are shown on the left with positive numbers specifying the range of run lengths. Retrograde run lengths are shown on the right with negative numbers specifying the range of values.

3.2.3 Rat KIF1A Does Not Disrupt Traffic of Non-KIF1A Associated Cargo

To determine if the decrease in DCV traffic in KIF1A knockdown cells was due to off-target effects of the rat KIF1A RNAi, I analyzed the movement of cargo known to associate with other motors. The motors kinesin-1 (KIF5B) and the KIF1A relative, kinesin-3 motor KIF1B have been implicated in the transport of mitochondria (Hirokawa and Noda, 2007). The dendrite specific transferrin receptor (TFR) (Silverman et al., 2001) is a receptor for iron bound transferrin, commonly associated with early endosomes (Moos et al., 2007). These have not been reported to be transported by KIF1A but by KIF16B (Hoepfner et al., 2005) or KIF5 (Schmidt et al., 2009). I filmed and analysed the movement of GFP-tagged mitochondria and GFP-TFR similar to that of DCVs in cells expressing control mouse KIF1A RNAi and rat KIF1A RNAi. Average mitochondria flux in control cells and KIF1A knockdown cells was 0.23 ± 0.04 min^{-1} and 0.27 ± 0.04 min^{-1} respectively with no significant difference between them ($p > 0.05$) (Figure 15 and Table 2). I found no significant difference ($p > 0.05$) in TFR flux between control and rat KIF1A RNAi cells either which was 2.13 ± 0.5 min^{-1} and 1.96 ± 0.4 min^{-1} respectively (Figure 17 and Table 2).
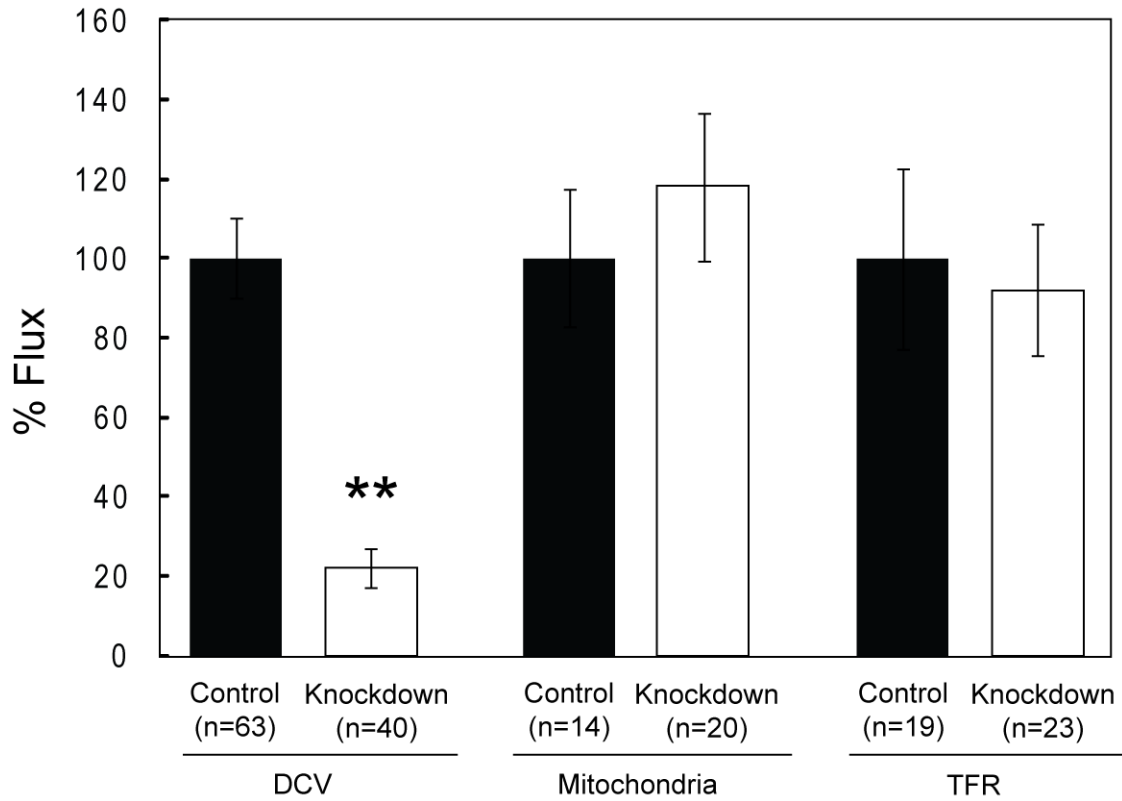
Figure 17. Organelle Flux in Knockdown Cells As a Percentage of Organelle Flux in Controls

The average flux of DCVs, Mitochondria and TFR in control cells expressing mouse KIF1A RNAi was taken to be 100% (darker bars). The flux of these organelles in knockdown cells was then calculated as a proportion of 100% (lighter bars) of their respective controls. Only DCV flux is significantly decreased in knockdown cells while mitochondrial flux and TFR flux are not affected. * $P < 0.05$, ** $P < 0.01$.

3.3 RNAi Resistant Rat KIF1A Rescues DCV Traffic

To provide further evidence for the primary role of KIF1A in DCV transport, I rescued DCV movement with a myc-tagged RNAi resistant KIF1A construct in knockdown cells. This construct was resistant to our RNAi because it contained 4 silent single nucleotide polymorphisms (SNPs) within the region targeted by the rat KIF1A RNAi. I filmed and analyzed DCV movement as previously described in neurons expressing rat KIF1A RNAi-GFP, DCV marker BDNF-RFP and the myc-tagged RNAi resistant KIF1A (Fig.18). Kymographs generated from movies of experimental cells rescued with the myc-tagged RNAi resistant KIF1A show a recovery of overall DCV flux compared to knockdown cells (Fig. 18C). Average overall DCV flux in these cells was 3.42 ± 0.06 min^{-1}, a value comparable to flux observed in controls ($p > 0.05$) but significantly different from DCV flux in knockdown cells not rescued with the RNAi resistant construct ($p < 0.01$) (Fig. 19 and Table 2). DCV anterograde velocity (1.5 ± 0.06 min^{-1}) and retrograde velocity (1.5 ± 0.07 min^{-1}) in rescued cells was slightly above control levels ($p < 0.01$ for both), (Fig. 20 and Table 2) but within previously reported limits (Okada and Hirokawa, 1999).
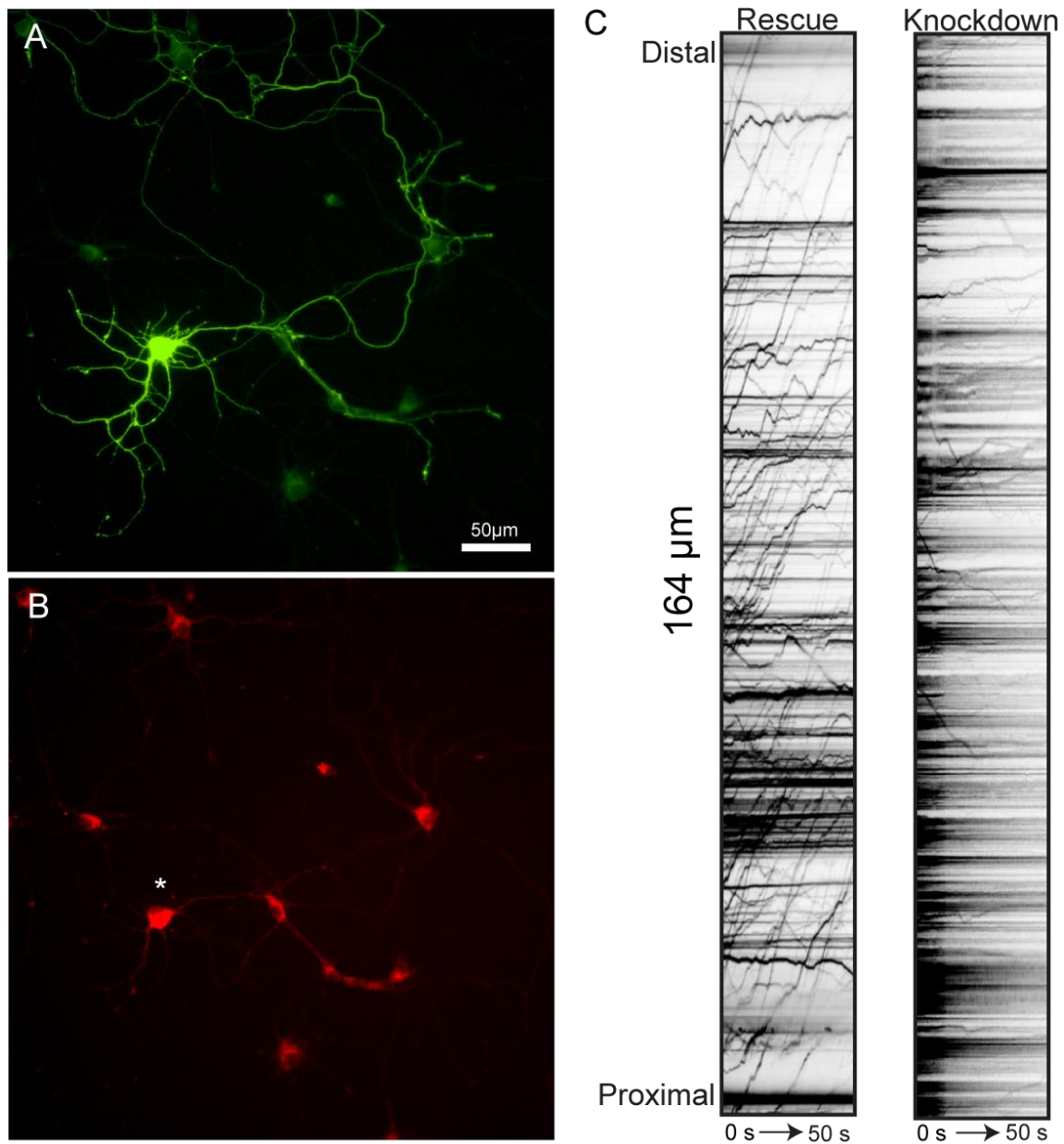
Figure 18. Cells Rescued with RNAi Resistant Rat KIF1A RNAi Show a Recovery of BDNF Transport

A, Live hippocampal neuron expressing GFP from the rat KIF1A RNAi containing pLox vector. *B*, The same cell as in (A) but now fixed, shown expressing myc-tagged RNAi resistant KIF1A (star). Panels A and B are magnified 200x. Fixed cells were stained with polyclonal anti-myc antibody (1:240) and Cy5 (1:500). Cells were considered to be transfected with myc-KIF1A if the intensity of the fluorescence in the cell body was twice above background fluorescence from other cell bodies at a magnification of 630x. *B*, Kymographs showing BDNF traffic in axons rescued with RNAi resistant KIF1A (left) and knockdown axons (right). Overall DCV flux is recovered in rescued cells compared to knockdown cells.
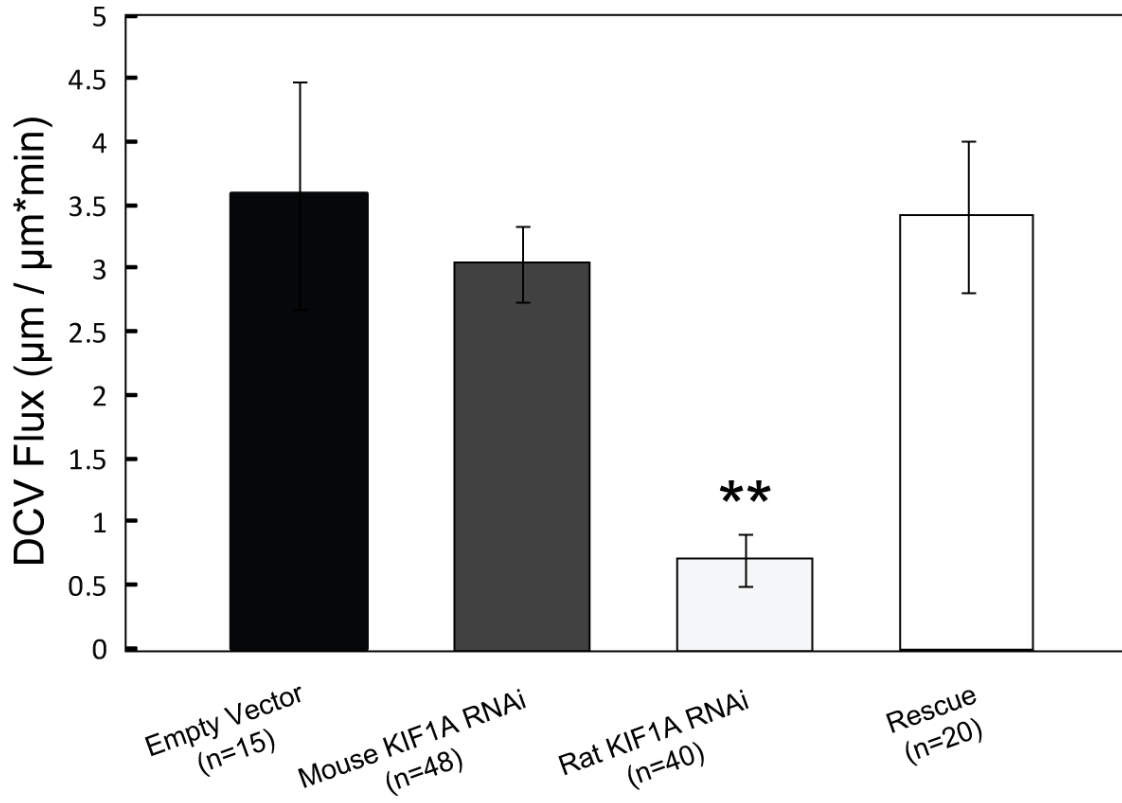
Figure 19. DCV flux is Recovered to Control Levels in Rescued Cells

DCV flux in rescued cells is comparable to DCV flux in control cell but is significantly greater than in knockdown cells. * $P < 0.05$, ** $P < 0.01$
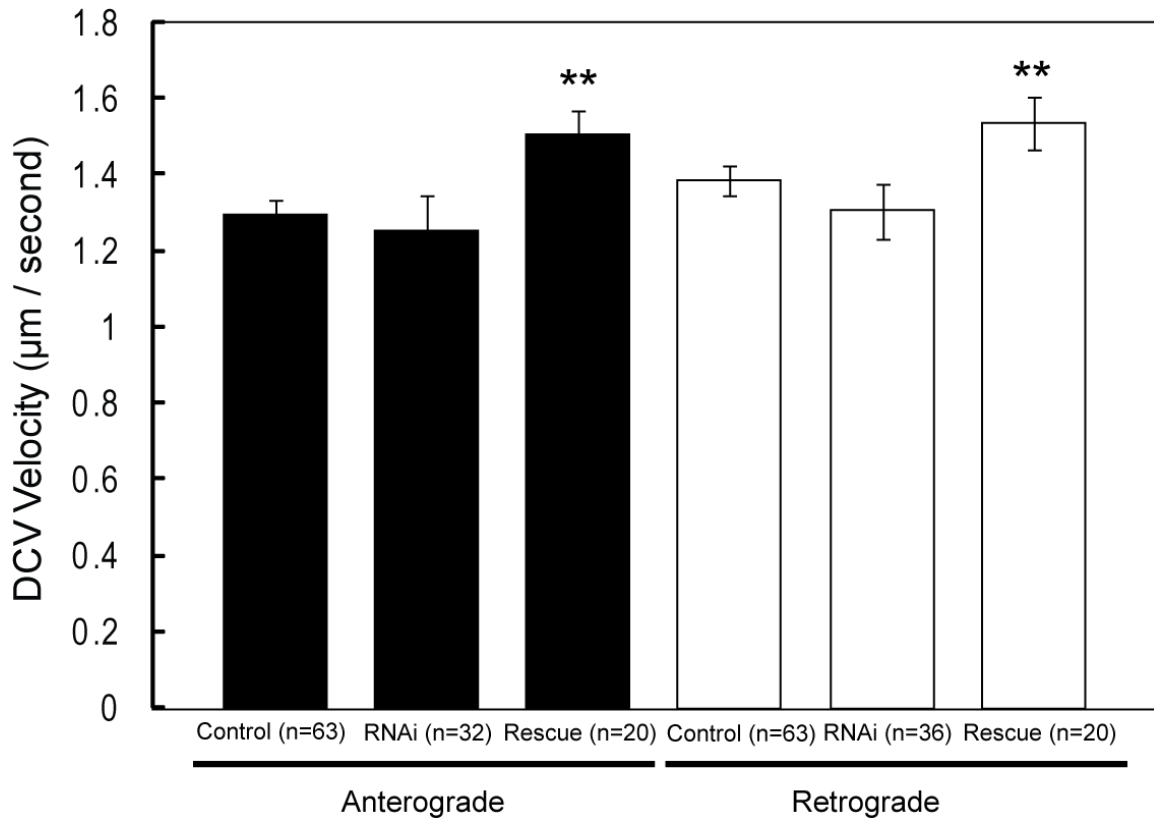
Figure 20. DCV Velocity in Control, Experimental and Rescued Cells

Anterograde and retrograde velocity in rescued cells is increased compared to controls and knockdown cells (RNAi).   ** $P < 0.01$.

Table 2. Quantification of Transport

	All Events	Anterograde Events	Retrograde Events
Flux (min $^{-1}$)			
Control[a]	3.17 ± 0.32	1.49 ± 0.16	1.68 ± 0.19
Rat KIF1A RNAi[b]	0.7 ± 0.16**	0.36 ± 0.09**	0.34 ± 0.07**
Rescue[c]	3.42 ± 0.58	2.18 ± 0.54	1.24 ± 0.11*
Mitochondria (control)[d]	0.23 ± 0.04	0.11 ± 0.02	0.12 ± 0.02
Mitochondria (knockdown)[e]	0.27 ± 0.04	0.14 ± 0.02	0.12 ± 0.02
TFR (control)[f]	2.13 ± 0.49	0.94 ± 0.25	1.19 ± 0.25
TFR (knockdown)[g]	1.96 ± 0.35	0.99 ± 0.17	0.97 ± 0.18
Velocity µm/s			
Control[a]	1.36 ± 0.04	1.3 ± 0.04	1.39 ± 0.04
Rat KIF1A RNAi[b]	1.29 ± 0.05	1.25 ± 0.06[α]	1.3 ± 0.06[β]
Rescue[c]	1.52 ± 0.06*	1.51 ± 0.06**	1.53 ± 0.07**
Mitochondria (control)[d]	0.3 ± 0.07	0.26 ± 0.05	0.32 ± 0.09
Mitochondria (knockdown)[e]	0.28 ± 0.05	0.24 ± 0.04	0.26 ± 0.06
TFR (control)[f]	1.37 ± 0.05	1.32 ± 0.09	1.35 ± 0.07
TFR (knockdown)[g]	1.39 ± 0.24	1.32 ± 0.22	1.45 ± 0.26
Run Length (µm)			
Control[a]	6.18 ± 0.29	6.16 ± 0.04	6.36 ± 0.56
Rat KIF1A RNAi[b]	5.46 ± 0.56	5.5 ± 0.7[α]	5.1 ± 0.4[β]*
Rescue[c]	5.39 ± 0.51	5.94 ± 0.75	4.72 ± 0.44
Mitochondria (control)[d]	5.75 ± 0.46	5.78 ± 0.83	5.22 ± 0.59
Mitochondria (knockdown)[e]	9.22 ± 1.05*	8.87 ± 1*	7.79 ± 1.2*
TFR (control)[f]	4.45 ± 0.34	4.12 ± 0.47	4.5 ± 0.43
TFR (knockdown)[g]	3.88 ± 0.51	3.8 ± 0.49	3.97 ± 0.52

a: n=63, b: n=40, c: n=20, d: n=14, e: n=20, f: n=19 g: n=23. α: The anterograde moving fraction in knockdown cells (n=32), β: The retrograde moving fraction in knockdown cells (n=36). *P<0.05, ** P<0.01 significantly different from controls.

4: DISCUSSION

4.1 Summary

The delivery of DCVs to presynaptic sites depends on active cytoskeletal-based axonal transport. The molecular motor KIF1A has previously been shown to associate with DCVs in cultured rat hippocampal neurons (Park et al., 2008) and to transport DCVs anterogradely in axons of *Drosophila* larva (Barkus et al., 2008) and *C. elegans* axons (Jacob and Kaplan, 2003). This thesis demonstrates that KIF1A is responsible for the anterograde axonal transport of DCVs in cultured rat hippocampal neurons because knocking down endogenous KIF1A had a severe effect on axonal DCV traffic and because exogenous, RNAi resistant, KIF1A was able to rescue the defect. The thesis also provides evidence for the association of KIF1A with DCVs during retrograde axonal transport even though KIF1A is a plus end directed motor.

4.2 KIF1A and DCVs Associate During Anterograde and Retrograde Axonal Transport

In this study KIF1A was observed to transport with the DCV marker BDNF during anterograde and retrograde axonal transport. Although, a significant difference was found between the percentage of BDNF, which transported with KIF1A and the percentage of KIF1A that transported with BDNF, the discrepancy

in colocalization percentages can be attributed to the partial solubility of KIF1A-GFP. When kinesins are not bound to cargo or microtubules they reside as soluble proteins within the cytoplasm (Reilein et al., 2001). The fluorescence from the soluble portion of the GFP-tagged KIF1A makes it difficult to make out distinct puncta due to the "haze" of background GFP (Fig. 5A, Fig. 5D and Fig, 6). BDNF, on the other hand has a much more punctuate appearance in the axon (Fig. 5B, Fig. 5E and Fig. 6). Additionally, *in vitro* studies demonstrate that as few as one or two motors are capable of moving a membrane bound organelle (Gross, 2004). Single molecule identification, which would be difficult using wide-field fluorescence microscopy, is not a goal of this study. Therefore, DCVs transported by single motors may be visible but the motors that transport them may not. The partial solubility of KIF1A results in generally fewer visible traces on KIF1A kymographs compared to BDNF kymographs. However, there is no reason to assume that BDNF associated KIF1A is any more likely to be visible on the KIF1A kymographs than non-BDNF associated KIF1A. Therefore, it is very likely that the reported percentage of KIF1A localizing with BDNF is more accurate than the percentage of BDNF co-localizing with KIF1A. Importantly, these results indicate that in the axon not only do KIF1A and DCVs move in tandem but that they do so almost equally in both the anterograde and retrograde direction. This data is supported by previous studies that recorded retrograde KIF1A movement in rat hippocampal neurons (Lee et al., 2003) and others that demonstrated a direct association of both plus and minus end directed motors with DCVs through dynactin (Park et al., 2008; Colin et al., 2008). This study is

the first, however, to quantify the bidirectional axonal co-transport of KIF1A and

DCVs in mammalian axons.   There was no statistical difference between

anterograde and retrograde DCV flux in control cells, knockdown cells, or rescue

cells (Table 2). The finding that KIF1A and DCVs transport together equally in

both directions is interesting because it indicates that an equal number of motor-

cargo pairs are transported back to the cell body as are transported to axon tips.

This relationship could be an example of the circulation of the reserve pool of

DCVs through distal axonal sites, observed in *Drosophila* neurons by

Shakiryanova et al., (2006). The fact that co-transport percentages did not

exceed 70% is likely a reflection of the additional synaptic vesicle precursor

cargo of KIF1A. BDNF has also been reported to associate with conventional

kinesin (Colin et al., 2008). This may be partially reflected by the lower

percentage of DCVs moving with KIF1A observed in this study.

The association of plus and minus end motors on the same cargo would

be a logical solution to the challenges a neuronal cell faces of transporting

components down the long stretches of axon. Such an association would allow

the cell to respond quickly to changing environmental cues by redirecting

organelle traffic during transport. In addition, it would provide the transiting

vesicle with an efficient means of navigating the obstacle-ridden world of the

axoplasm and so allow it to reach its destination sooner. With both motors

attached a vesicle could back up to navigate around the obstacle (Ross et al.,

2008). Consistent with this notion the reversal of DCVs has been documented

during axonal transport in hippocampal cultures (Kwinter et al., 2009) and *C.*

elegans (Zahn et al., 2004) and *Drosophila* (Shakiryanova et al., 2006). It seems most likely that such fine-tuning of transport can best be accomplished if the attached plus and minus motors are regulated by a switching mechanism such as dynactin. Emerging evidence supports the hypothesis that dynactin is indeed such a mechanism (Deacon et al., 2003; Colin et al., 2008). Although microtubule minus end kinesins could theoretically be responsible for the retrograde transport observed there is evidence that indicates that dynein is the more likely candidate. Overexpression of dynactin subunits impairs bidirectional axonal DCV transport (Kwinter et al., 2009) and Park et al., (2008) immunoprecipitated dynein associated intermediate chain along with the p150 component of dynactin together with DCV associated proteins.

4.3 KIF1A Transports DCVs in Hippocampal Neurons

This thesis provides *in vivo* evidence for the primary role of KIF1A in the axonal transport of DCV in mammalian neurons. Knocking down endogenous expression of KIF1A in hippocampal cultures resulted in a near absence of DCV axonal transport in both the anterograde and retrograde directions. The defect was severe enough to decrease DCV axonal flux by over 77%, a percentage that reflects the proportion of KIF1A co-transporting with BDNF anterogradely and retrogradely (70% and 64% respectively) (Fig. 8). These results agree with those obtained by Barkus et al., (2008) who also report an over 67% decrease in anterograde DCV transport in *Drosophila UNC-104* mutants. Indeed, the decrease recorded in this study was so severe as to imply that KIF1A is the primary motor responsible for anterograde DCV axonal transport in hippocampal

neurons. Importantly, the transport of other, non-KIF1A associated cargos, TFR and mitochondria, was in no way affected in KIF1A knockdown cells, implying that the decrease in DCV flux observed is indeed due to a depletion of endogenous KIF1A levels (Fig.17 and Fig. 19). This result is supported by a previous study that also did not detect a change in mitochondrial transport upon KIF1A disruption (Barkus et al., 2008). The absence of a disruption of mitochondrial traffic in KIF1A knockdown cells is particularly relevant considering that one of the motors that transports mitochondria is KIF1B (Boldogh and Pon, 2007), a motor that bears some amino acid sequence homology to the C-terminus of KIF1A (Hirokawa and Takemura, 2004). Anterograde and retrograde DCV transport velocities reported by previous *in vivo* studies with hippocampal neurons (Park et al., 2008) and cortical neurons (Colin et al., 2008) was below 0.5 μm / second. The velocity data presented here is significantly greater (1.0-1.5 μm / second) and is more in line with in vitro velocities obtained for KIF1A (Okada et al, 1995) and *UNC-104* velocities obtained from *Drosophila* neurons (Barkus et al., 2008; Shakiryanova et al., 2006). This is likely a result of the unique method of data acquisition used in this study. Videos of axonal transport consisted of 200 frames of consecutive exposures of 250 ms which allowed for the detection of fast moving particles. Vesicles travelling less than 2 μm were omitted from analysis as such non-processive movement is not characteristic of KIF1A transport. This method was used to image DCVs by Kwinter et al., (2009) in hippocampal neurons who report DCV velocities similar to those reported in this study. The velocity of the moving fraction of DCVs in knockdown cells was

not significantly different from controls indicating that this fraction is KIF1A associated. The decrease in retrograde run length in knockdown cells may be a result of the impaired ability of dynein to navigate around obstacles without the presence of KIF1A due to improper steric coordination of the motors. It is possible that the moving fraction of DCVs observed in knockdown cells could be transported by a different kinesin motor, such as conventional kinesin as was previously suggested (Colin et al., 2008). The DCV run lengths reported here (Table 2) are below those previously reported for KIF1A in live hippocampal neurons (11 μm) (Lee et al., 2003). However, the DCV run lengths reported here are in agreement with those of a processive motor and reflect those reported for DCVs previously (Kwinter et al., 2009). The discrepancy in run length may be a result of the specific association of KIF1A with its DCV cargo. For instance KIF1A binds directly to synaptic vesicle precursors with residues in its PH domain (Klopfenstein and Vale, 2004), while KIF1A association with DCVs may be mediated by dynactin (Park et al., 2008; Kwinter et al., 2009).

4.4 Exogenous KIF1A Rescues DCV Flux

The introduction of exogenous RNAi resistant KIF1A into knockdown cells rescued axonal anterograde and retrograde DCV transport. DCV flux was recovered to control levels in rescued cells while anterograde and retrograde DCV velocity exceeded that seen in controls. Anterograde run lengths were recovered to control levels but retrograde run lengths remained significantly lower in rescued cells. The changes in transport velocity may be a result of KIF1A over-expression and would support the tug-of-war hypothesis of

bidirectional transport (Fig. 4). Either way the anterograde velocity observed in rescued cells does not exceed those previously reported for kinesin (Okada et al., 1995). The recovery of the DCV transport defect in experimental cells with the recombinant KIF1A implies that lack of KIF1A alone is responsible for the significant decrease in axonal DCV transport observed. In addition, it is important to note that as both anterograde and retrograde DCV transport was inhibited in knockdown cells, so both anterograde and retrograde transport were recovered in rescued cells. This phenomenon fits perfectly with the near identical levels of anterograde and retrograde KIF1A and BNDF co-transport indicating that anterograde and retrograde transport is linked. Perhaps proper DCV transport depends on the stoichiometric levels of plus and minus motors, which makes sense if they are both needed to transport individual cargos. Importantly, the knockdown and recovery experiments show that KIF1A is the primary mammalian kinesin motor responsible for axonal DCV transport in hippocampal neurons.

4.5 Conclusions and Future Perspectives

Neuronal survival and function are dependent on proper DCV cargo delivery to presynaptic axonal sites. Defects in such motor-cargo interactions can have serious implications for nervous system function and can involve different components of motor-cargo interaction machinery. For instance Huntington's disease (De Vos et al., 2008) and ALS may be caused by defects in motor-cargo linkage (Stokin and Goldstein, 2006) while CMT 2A is result of defective motor function (Zhao et al., 2001). This study identifies KIF1A as the

primary motor for the axonal delivery of DCV cargos to axon terminals. Identification of motors responsible for the delivery of pre-synaptic cargo will contribute to the understanding of neuronal function and perhaps shed light on the causes of diseases.

Future experiments should be geared towards understanding the mechanisms by which KIF1A selects and binds its known cargos, and perhaps a search for other, yet undiscovered cargos. Specifically, biochemical experiments such as co-immunoprecipitation assays could be performed to demonstrate a physical interaction between KIF1A, DCVs and perhaps dynactin.

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