The Role of Kinesin-3 Family Members in Dendrite-Selective Transport

By

Brian Vincent Jenkins

A Dissertation

Presented to the Neuroscience Graduate Program

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

May 2012

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of
BRIAN JENKINS
has been approved on May 16th, 2012

_____
Advisor, Gary Banker, PhD

_____
Member and Chair, Peter Gillespie, PhD

_____
Member, Stefanie Kaech Petrie, PhD

_____
Member, Linda Musil, PhD

_____
Member, Philip Copenhaver, PhD

_____
Member, Anthony Paul Barnes, PhD

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Movies

Abstract

The ability of a cell to transport cargo vesicles to their correct destination is called selective transport. Neurons, like other cells, require this system in order to develop and function properly. The neuron's primary function is to send and receive signals. In order to do so, neurons have developed specialized domains, namely, the axons and dendrites. Both domains require a specific complement of proteins in order to carry out its function. The focus of this work is the underlying mechanism that is responsible for selective vesicle transport in neurons.

Kinesin-mediated transport has been implicated in the selective transport of dendritic vesicles, but the mechanism for this is not understood. In this thesis, I present evidence that KIF13B from the Kinesin-3 family is a dendrite-selective kinesin. Expression of GFP-tagged Kinesin-3 family members revealed that while all constructs labeled moving vesicles, KIF13B was unique in that it was the only family member to label vesicles that trafficked exclusively in dendrites. KIF13B is the first kinesin to demonstrate this transport characteristic and further investigation may yield insight toward a mechanism for dendrite-selective transport.

I investigated the contribution of the motor domain of KIF13B and its other regions in selective transport using a cell-based transport assay. I found that the motor domain is not sufficient for dendrite selective transport. In addition, I found that other regions within KIF13B were not able to confine transport to the

dendrites. This work points towards the conclusion that KIF13B must act in concert with other elements in order for it to act as a selective dendritic motor.

To investigate what these other elements may be, I collaborated with other members of the Banker lab to develop a novel assay that identified the kinesins that mediate dendrite-selective transport. We prepared a library of “split kinesins”, comprising an axon-selective kinesin motor domain and a series of kinesin tail domains that can attach to their native vesicles. When the split kinesins are assembled by chemical dimerization, bound vesicles are misdirected into the axon. Three Kinesin-3 family members--KIF1A, KIF13A, and KIF13B--interacted with dendritic vesicle populations in the split kinesin assay. KIF1A and KIF13A also bound vesicles that translocate into the axon. These results demonstrate that kinesins play an integral role in dendrite-selective transport and that their transport selectivity is differentially regulated when they bind different vesicular cargoes. Identifying these kinesins and further exploring the proteins they interact with will help in our understanding of dendrite-selective transport and perhaps lead to a mechanism by which this is regulated.

Finally, I will discuss specific models that could account for the selectivity of KIF13B-mediated transport, and propose experiments to test the accuracy of these models.

Acknowledgements

This is the culmination of a journey that began in 7th grade during my life science class which sparked my interest in the biological sciences. The long journey to the Banker lab and earning a Ph.D. would not have been possible without the help of many people along the way.

First and foremost, I would like to thank Gary for not only allowing me to join his lab and carry out this work but also for his seemingly unlimited amount of patience and guidance as a mentor while I made my way through graduate school. I am grateful the many, many hours he set aside for me to listen to my ideas, talk out experiments, discuss results and assist with writing. Thank you Gary.

I would also like to thank Stefanie Kaech for co-mentoring me and preparing me for all things related to kinesins and live-cell imaging. For always making time to discuss experiments, and becoming a great friend along the way while keeping me well fed with the help of her husband Phil.

I would like to thank the entire Banker lab past and present for making it a pleasure to come into work. Chun-fang Huang, Jennifer Petersen and Cheng Fang for constant discussion and advice throughout my PhD. For Julie Luisi and Barbara Smoody for their expert technical assistance. For Helena Decker and Marvin Bentley as they provided lively discussion about how kinesins might or might not be responsible for dendrite-selective transport and listening to my crazy

ideas while keeping me on track to graduate. Helena contributed greatly to making the figures presentable and Marvin for making this thesis readable.

I am thankful to my committee for not just challenging me and my ideas but for treating me as a peer. I always got the impression that it was not them versus me but rather us versus science with them as guides through this work. Thank you Peter, Philip, Linda, Stefanie and Gary.

I am indebted to professors Shelley Halpain and Steve Kay while I was at The Scripps Research Institute. I began this career volunteering to wash dishes in Shelley's lab. Soon after, I was hired part-time to help run the lab and given an independent project, under the guidance of Leif Dehmelt. After graduating, Steve hired me as a technician but gave me freedom to work alongside a talented post-doc, Jessie Chu, where I gained experience guiding a project.

My time at Scripps instilled within me a strong work ethic and provided experiences and exposure to how science is conducted which allowed me to succeed in graduate school. I am grateful for my time spent working with Leif Dehmelt, Jessie Chu, Barbara Calabrese, Julia Braga, Eric Hwang, Kathy Spencer and Tom Schultz. Each contributed greatly to my decision to attend and complete graduate school. And to Elena Gastelum and Toni Lestelle for making my time at TSRI most enjoyable.

I would like to thank my family for keeping me grounded, focused and well fed. My mom for constant support in all matters and reminding me that if two people think the same then one of them is unnecessary. My dad for supporting

me and believing in me to achieve this goal. For my brother and his family for their support and keeping me in touch with in the ‘real world’ and always making me feel welcome when I needed a break from the lab.

I am thankful to Karin, for opening my eyes to the world and making me a more independent and well-rounded individual. For her patience and limitless support during my Ph.D. and my struggles to write this thesis., For reminding me there is more to life than benchwork, as we went on many adventures together in Chile and throughout Oregon and Washington to climb, camp, hike,snowboard and summit mountains.

I would like to thank my classmates Delia, Derek, Aya, Isabelle, Art, Rebecca, Julia, Cadence, Aaron and Kimmy for camaraderie through the years – we had some great times and knowing we are not struggling alone always brought us together. For Quicksilver, my drinking team with a soccer problem…GOOOOO QUICKSILVER!

I would like to thank Gary, Marvin Bentley, Rachel Dresbeck and my writing group Kalmia, Greg and Sarah who without them this thesis would not have been written.

Chapter 3 is a version of a paper to be submitted that was a collaborative effort from Marvin Bentley Ph.D., Helena Decker Ph.D. and I.

Figure 4 in Chapter 3 was contributed by Helena Decker Ph.D.

Thank you all.

Chapter1: Introduction

A longstanding question in the neuroscience field is how kinesin-mediated transport is involved in the development and maintenance of neuronal polarity. More specifically, what is the mechanism underlying selective transport of dendritic vesicle populations? In this dissertation, I will provide evidence for a novel dendritic kinesin, and describe a technique to identify kinesins mediating transport of dendritic cargos. These studies may provide clues to understanding the mechanism underlying dendrite-selective transport.

Neuronal polarity

Neurons are typically divided into two domains: the axonal and somato-dendritic. These domains have developed unique structures to carry out their individual functions. Structurally, a neuron's large dendritic arbor enables input reception from many axons while its long, branching axon contacts many neurons that are long distances away. Functionally, its two separate domains, somato-dendritic and axonal, carry out two specific functions of receiving and sending electro-chemical signals, respectively. These two separate and unique domains that make up the neuron give rise to the term "neuronal polarity".

How neurons develop and maintain this polarity is a longstanding question in neuroscience. One model system suited to study this question is cultured hippocampal neurons [2]. In this system, neurons are cultured at low density on coverslips and can remain alive and healthy for up to six weeks. Culturing makes it possible to visualize an individual neuron under a microscope as it develops. Dotti et al. cataloged the development of neurons using this culture system and found that neurons pass through specific stages of development to mature into a synaptically active network [1]. In culture, neurons go through five stages of development (Figure 1) [1]. In stage 1 (1-2 hours after attaching to the coverslip) the neurons have a circular cell body surrounded by a lamellipodia. Stage 2 (3-12 hours post attachment) begins when appendages form and extend from the cell body. These appendages, termed “minor processes” or “neurites”, are morphologically and molecularly similar; thus, the neuron is not yet polarized. During stage 3 (18-36 hours), polarization arises when one of these neurites

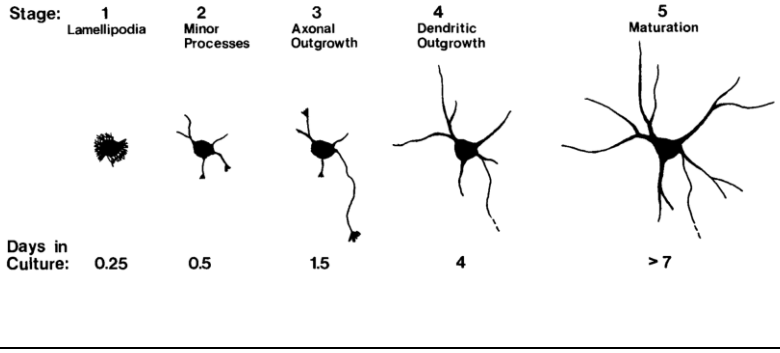


Figure 1. Illustrations of the 5 stages of neuronal development in culture. Adapted from Dotti et al. [1]

spontaneously grows faster and becomes longer than the others, and this neurite becomes the axon. At stage 4, 3-6 days in-vitro (DIV), axonal outgrowth continues, the axon initial segment (AIS) is fully formed, while dendrites also

grow longer and taper toward their end [1, 3]. Stage 5 (~>14 DIV) is characterized by the appearance of dendritic spines. Neurons have been shown to develop in a similar manner in the developing cortex [4]. These early studies of neuronal development in culture suggest that neurons have an intrinsic mechanism for developing and maintaining polarity. This culture system is ideal for studying the effects of a particular protein on neuronal polarity because it is easily manipulated by techniques such as immunostaining, ectopic expression of GFP-fused proteins and RNAi knockdown [5, 6]. In short, neuronal cultures have proven to be a very useful tool to study the development and maintenance of polarity. For these reasons, I used cultured hippocampal neurons as the model system in my thesis work to study the kinesins that mediate transport in dendrites.

In order for the neuron to perform its duty of receiving and sending signals, specialized proteins are required to be in the correct domain. For example, dendrites receive chemical signals through membrane-bound proteins in the post-synapse region that respond to neurotransmitter release. This response is mediated by specialized receptors and channels that not only depolarize the membrane to facilitate action potential firing but also to influence myriad specialized proteins that regulate cytoskeleton rearrangement, receptor trafficking, and signaling cascades [7, 8]. Furthermore, specialized scaffolding proteins are needed to anchor the receptors and signaling molecules in the post-synaptic compartment [9]. In order for axons to send signals, specialized machinery is required at the pre-synaptic terminals for synthesizing

neurotransmitters, packaging them into vesicles, and releasing these vesicles upon membrane depolarization [10]. Neurons also need specific sodium channels (Nav1.6) to be delivered to the axon initial segment and nodes of Ranvier to propagate an action potential [3]. Thus, the neuron must have the correct pre- and post-synaptic proteins to receive, interpret and propagate action potentials. If proteins were delivered to the wrong domain, then the neuron might be unable to carry out its functions efficiently or at all. It is therefore necessary for the cell to have a mechanism in place to ensure the proper delivery of proteins so the cell can function as intended.

One such mechanism is selective microtubule-based transport. Selective transport is defined as the transport of a specific protein or protein complex to a distinct location within the cell and not to another. An example is the transferrin receptor (TfR), an integral membrane-bound protein that is found abundantly in the dendritic membrane and in dendritic vesicles but that rarely enters the axon [6]. This suggests that the cell has a mechanism in place to restrict dendritic vesicles from entering the axon. Upstream of transport, the mechanism for sorting proteins into the correct vesicle is fairly well understood [11, 12]. In contrast, the mechanism of how vesicles are selectively transported into one domain and not another is less well understood and is the central focus of this dissertation.

Selective transport and delivery is not the only mechanism by which neurons establish compartmentalization. Proteins embedded in the membrane can diffuse throughout the membrane if not attached or anchored in place. For

example, the specialized compartment in the axon known as the axon initial segment, has membrane-bound sodium channels that are anchored to the cytoskeleton to prevent their diffusion throughout the axon and somatodendritic domain. This diffusion barrier prevents mixing of membrane proteins between axons and dendrites [13, 14].

Kinesins and vesicle transport

The transport of proteins from their site of synthesis to their final destination is mediated by microtubule-based transport, which is carried out by the molecular motors kinesins and dyneins. With the exception of a few C-terminal motor kinesins [15], kinesins transport to the plus end of microtubules. Dynein transports to the minus end of microtubules. In axons, 90% of the microtubules are oriented with their plus-ends distal to the cell body [16]. Thus, kinesins are suited to carry out long range axonal transport toward the axon terminals (anterograde), and dynein is responsible for most of the transport toward the cell body (retrograde). Inactive dyneins must transport on axonal vesicles to reach the axon terminal in order to then return vesicles, such as autophagosomes and signaling endosomes, to the soma [17]. Both kinesins and dyneins have been implicated in dendritic transport of vesicles, where microtubule polarity is mixed [18-20]. Since the delivery of proteins from their sites of synthesis in the cell body to the correct intracellular destination is mediated principally by kinesins, kinesins will be the focus of this discussion.

The Kinesin Superfamily

All cells use kinesins to transport vesicles to the plus end of microtubules. Kinesins comprise two general domains; the motor domain and the cargo binding or tail domain (see Figure 2) [15]. The motor domain hydrolyzes ATP and moves in a 'hand-over-hand' manner along microtubules [22]. The motor domain interacts with tubulin through microtubule-binding regions called "loops". Loops from different kinesins have been shown to influence the ability of a kinesin to move in the axon and dendrites [23]. Tubulin modifications have also been shown to influence kinesin transport [24]. The kinesin tail domain has specialized regions that bind specific adaptors that mediate interactions with vesicles and other cargo [15, 25, 26].

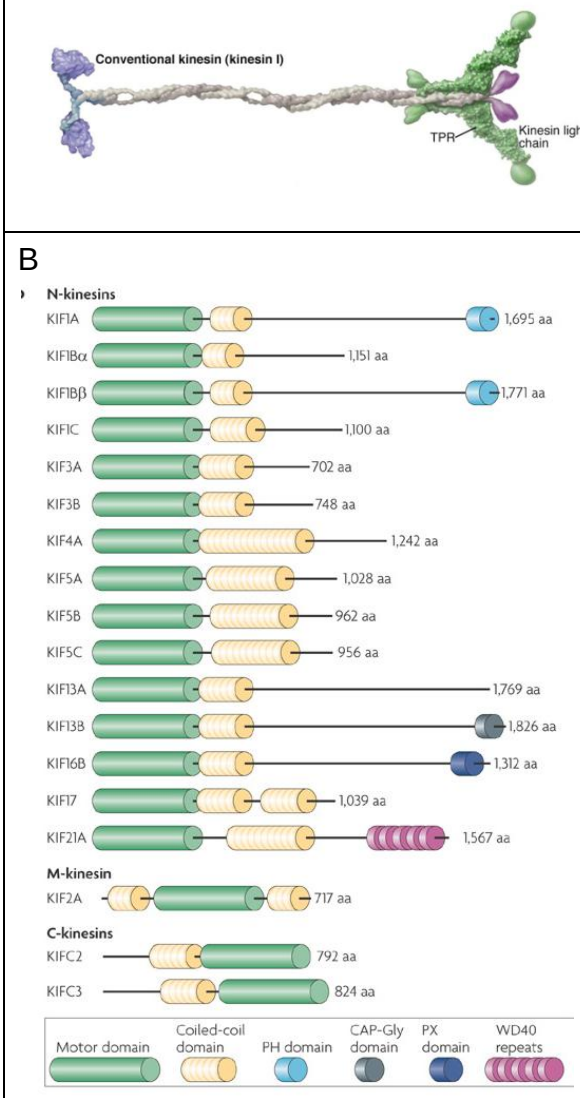


Figure 2. Kinesin structure and diversity. A, Cartoon of conventional kinesin (Kinesin-1) with Kinesin Light Chains. Adapted from The Molecular Toolbox [15] B. Cartoon representation of members of the kinesin superfamily and their domains adapted from Hirokawa [21]

The Kinesin superfamily comprises many subfamilies [25]. The sub-families are organized based on sequence similarities between the motor domains. In contrast, the tail domains of different kinesins,can diverge significantly, even within a family . This divergence allows each kinesin to interact with a unique set of cargo proteins (See figure 2B). Just as divergent as the tails is the nomenclature. Families of kinesins are listed numerically as Kinesin-1, -2 etc… In mammals, members of a particular family are called KIFs, with the numbers and letters after the KIF prefix designating different genes. The family number and the KIF number are not related and letters after a KIF indicate different genes. Splice variants are indicated by a lower-case Greek letter. For example, KIF1B is from the Kinesin-3 family and has 2 splice variants (KIF1Bα and KIF1Bβ) [28].

There are many different kinesins expressed in hippocampal neurons (see figure 3B dendrogram [27]). There are also many different vesicle populations within a cell, and each vesicle has as specific route to travel [29, 30]. The diverse tails of kinesins not only allow for transport of many vesicle populations but also allow them to participate in many cellular roles, including mitosis, microtubule

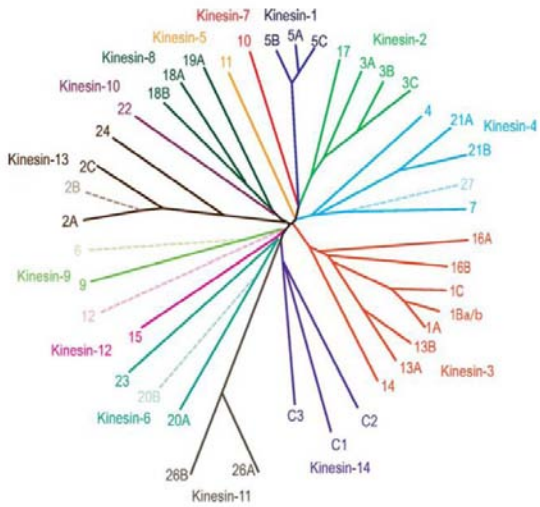


Figure 3: Dendrogram of kinesin families. Solid lines indicate kinesins expressed in hippocampal neurons. Adapted from Silverman et al. [27]

transport, scaffolding attachment, and anchoring of the ER and Golgi [31, 32]. This diversity in kinesins and cargos makes it difficult to answer the question of which kinesin transports which cargo. This is further compounded by the fact that kinesins can have adapter proteins to further expand their repertoire for more cargos. The next section discusses and presents examples of kinesin-cargo interactions.

Kinesin and cargo interactions

The large majority of kinesins attach to cargo vesicles through adaptor proteins. In some cases, the adaptor protein is specific for the kinesin. For example, kinesin light chain (KLC) is specific for the Kinesin-1 family, and kinesin associated protein (KAP) is specific for members of the Kinesin-2 family [33, 34]. In other cases, the adaptor proteins are scaffolding proteins implicated in other cellular functions [35-37].

Kinesin-1 or conventional kinesin is the best understood family member. Kinesin-1 exists as both a dimer and a tetrameric protein [31]. Kinesin-1 comprises two heavy chains which form a dimer, and two kinesin light chains may too be components of Kinesin-1. Kinesin-1 binds some cargo/adapter proteins directly through the C-terminal region of the heavy chain and to others through its light chain. There are three isoforms of Kinesin-1 and three isoforms of KLC, each of which has multiple splice variants [38]. This combination of isoforms and splice variants may contribute to the varied cargo proteins of Kinesin-1. Kinesin light chain typically binds to its cargo through its

tetratricopeptide repeats domain (TPR) [31]. For example, Verhey and colleagues used KLC as bait in a yeast-2-hybrid screen and found it interacted with JNK interacting proteins 1,2, and 3 (JIP1,2,3) [37].

Not all Kinesin-1 cargoes link via KLC. The kinesin heavy chain binds to mitochondria through a different adapter protein called Milton [39]. Kinesin-1 is not the only kinesin to link its cargo through kinesin-specific adapter molecules. Kinesin associated protein 3 (KAP3) is an adapter protein for the heterodimeric Kinesin-2 family member KIF3A-3B. KAP3 binds to the C-terminus of the KIF3A-3B dimer to form a heterotrimer and has been shown to interact with fodrin, an actin binding protein [34].

Other kinesins bind to vesicles via scaffolding proteins that have multiple functions. For example, KIF13A, a member of the Kinesin-3 family, associates with mannose-6-phosphate receptor-containing vesicles via a linkage through β1 adaptin of the AP1 complex [40]. The AP complexes are involved in protein targeting to different intracellular membrane compartments [11].

In a few cases, kinesins have been shown to interact directly with the vesicle through interaction with certain phospholipids. KIF1A, a member of the Kinesin-3 family, contains a C-terminal pleckstrin homology (PH) domain capable of binding to PIP2. Klopfenstein et al. tested this interaction by transferring the PH domain of KIF1A to a Drosophila kinesin named NCD and demonstrated it was able to transport PIP2 vesicles [41] . While this particular PH domain is sufficient for transporting membranous vesicles, it is unclear if this interaction

serves a supporting role for cargo transport by stabilizing the kinesin-cargo interaction or is actually how KIF1A interacts with vesicles. Since, KIF1A has not been shown to bind indiscriminately to all PIP2 in the cell, further regulation for attaching to the vesicle must be present. KIF16B, also from the Kinesin-3 family, was shown to bind to PI(3)P via a PhoX homology (PX) domain located in its tail. This PX domain was demonstrated to be necessary for KIF16B to associate with early endosomes, as mutations within the PX domain disassociated KIF16B from endosomes [42].

KIF1A does not exclusively bind to cargo through its PH domain. The stalk domain was shown to bind to Liprin-α, a scaffolding protein involved in AMPA receptor targeting. The KIF1A homolog in C. elegans, Unc104, was shown to be involved in transport of synaptic precursor proteins [43]. Hirokawa's group further investigated the role of Kinesin-3 family members KIF1A and KIF1Bβ in the transport of synaptic precursor proteins in mammals [44]. The authors used a region of the stalk domain of KIF1Bβ as bait in a yeast-2-hybrid screen to identify interacting proteins and identified the C-terminal death domain of DENN/MADD, a Rab3 GD-GTP exchange factor. Using several methods, they provided evidence that KIF1Bβ binds to Rab3 vesicles through DENN/MADD. Overall, these experiments support the model that KIF1Bβ links to Rab3-containing vesicles through DENN/MADD, and that DENN/MADD binds preferentially to the active form of Rab3-GTP. This suggests that the cargo-kinesin interaction may be regulated by the nucleotide state of Rab3. This is

important for the present work because the states of protein modifications and their influence on the kinesin-cargo interaction will be discussed later.

In summary, kinesins typically interact with their cargo through an adaptor protein or complex. A tremendous amount of research has been directed towards elucidating the relationship between kinesins and their cargo. These studies provide evidence as to which kinesin interacts with which cargo, a first step toward investigating how each may contribute to selective transport. However, these descriptive papers do little to address how the kinesin-cargo complex reaches its final destination or is excluded from specific intracellular domains.

Kinesins and dendritic cargos.

Methods to identify kinesin-cargo interaction

This thesis focuses on the mechanisms kinesin-mediated, dendrite-selective transport. In this section, I will present evidence from the literature that certain kinesins bind to specific dendritic cargos. I will emphasize the findings and techniques used in several papers to identify kinesins that carry dendritic cargos. This information is important because if kinesins are involved in regulating dendrite-selective transport, then identifying interactions between a kinesin and a dendritic vesicle may provide clues to understanding this mechanism.

Authors have used different methods to identify kinesins that interact with dendritic proteins. Some of the methods used in conjunction with the right controls lead to convincing results to support the authors' hypotheses, whereas other methods only lead to implied relationships between the two proposed interacting proteins. First, I will outline the major techniques used and the controls needed to substantiate the authors' claim of interaction. I will then critique the findings in some key papers in the field that used some of these techniques.

The majority of authors used a yeast-2-hybrid assay to identify candidate interacting proteins. Typically, the kinesin tail is used as the bait. Although this is a widely used and well-accepted technique to test for interacting proteins, it suffers from significant limitations. One main limitation to this technique is the amount of false positives obtained based on over-expression of the proteins in yeast. Because of this problem, it is necessary to validate these results with an immunoprecipitation (IP) as a control to confirm the interaction, as described below.

Another problem associated with this technique is the prevalence of false negatives. Some modifying enzymes (kinases or glycosylases) may not be expressed in yeast, and interactions may not be detected if these modifications are necessary for the interaction. Also, since the proteins used in this assay are fusion proteins, interactions may be difficult to obtain if the N- or C-termini are necessary for interaction. Another issue is that these interactions take place in the nucleus. If the protein and/or bait cannot enter the nucleus or resides in

another compartment, then interactions may not be discovered. A yeast-2-hybrid screen is a useful tool to identify candidates, but must be followed by a second assay to confirm the interaction.

This second assay to follow up on a yeast 2-hybrid is usually a co-immunoprecipitation of the interacting proteins from cell lysate. This approach depends heavily on a specific antibody to the protein of interest. In some cases non-neuronal cell lines have been used, which may yield different results from proteins expressed in vivo or in cultured neurons. Also, if the protein-protein interaction is electrostatic, it is dependent on the ionic concentration of the lysate buffer, which may differ from that of the intracellular environment. Controls for such an experiment should include another kinesin, preferably from the same family, to identify whether the binding is specific for the kinesin in question. It is also important to know how much protein was used as input so that the efficiency of the interaction or the binding capacity of the protein can be evaluated. Since kinesins may interact with a membrane-bound vesicle protein or a cytosolic protein, the use of a vesicle fraction for an immunoprecipitation is a useful technique to show that the kinesin can bind to a protein complex on an endogenous vesicle [31]. This biochemical approach suggests two proteins can interact, and in conjunction with yeast-2-hybrid, provide strong evidence that an interaction can occur. While the interaction may likely exist in the cell, these techniques do not resolve the biology of the interaction nor do they address the question of whether the kinesin transports the given cargo as this is not a

transport assay. It could be that the interaction directly or indirectly inactivates the kinesin through binding.

In most papers, the authors use cells or tissue to test whether the two proteins of interest have an overlapping distribution based on immunolocalization. Similar to an immunoprecipitation, this technique is dependent upon the availability of a specific antibody that produces little background. Antibodies often produce speckles of varying sizes, which makes identifying real proteins on vesicles difficult because they also appear as speckles. Moreover, demonstration that two specific antibodies yielding an overlapping signal still does not prove an interaction between the two proteins exists as the two signals may overlap in the x-y plane but could be separated in z. If a region within a protein is known to mediate the interaction, then expressing a mutant protein without this region should reduce overlapping signal. This important control is, however, often not done, and the authors often simply interpret overlapping signals as proof of an interaction. Overall, co-localization of a kinesin with a cargo is not strong evidence that the kinesin transported the cargo.

Disruption of kinesin function either by reducing expression with RNAi or by using a dominant negative kinesin construct are also widely used techniques to implicate a cargo-kinesin interaction. In the case of a knockdown, a decrease in the amount of the kinesin should result in less of the cargo reaching its destination. The dominant negative approach aims to disrupt all cargo transport by a certain kinesin by substituting a non-transporting kinesin. In practice,

however, the kinesin may be involved in more than one cellular process, the disruption of any of which could alter the pathway of the cargo without directly affecting its transport. In order for membrane proteins to reach the plasma membrane they must be properly folded, modified, packaged in the ER and Golgi, and then transported to their correct plasma membrane domain, These processes involve many proteins [11, 45, 46]. Once in the membrane, scaffolding proteins must keep it in place until it is removed and re-sorted or degraded. Kinesins have been shown to interact with membrane scaffolding proteins and endosomal proteins such as Rabs. Disruption of these interactions may affect the cargo proteins half-life in the cell membrane, turnover, and/or endosomal sorting [29, 30]. It is therefore difficult to pinpoint exactly where in the pathway the disruption of the kinesin alters the trafficking of the cargo. In the case of RNAi knockdown, a rescue experiment should always be used to show specificity. Even if the rescue is successful, the experiment may still not pinpoint where in the pathway the rescues worked if the biological consequences of the knockdown or dominant negative is unknown.

In summary, the methods described above, even with their faults, have provided clues as to the identity of dendritic proteins that interact with kinesins. To demonstrate whether a kinesin transports a certain cargo, live-cell imaging of them moving together on the same vesicle is the best available evidence and should be a requisite. Chapter three of my dissertation describes a new technique using live cell imaging to identify the kinesins that interact with dendritic cargos.

Examples of kinesin-cargo interactions

KIF17 was found to bind to two dendritic cargoes using three different methods. In the first example a yeast-2-hybrid was used to search for interacting proteins and confirmed by another group using a chimera kinesin approach [19, 47]. In the second example the authors used an unbiased screen of dominant negative kinesins [48]. While these groups show elegant biochemical protein binding results, the implications for transport are not clear.

KIF17 was first suggested to be a dendritic kinesin based on immunostaining that overlapped with MAP2 and not the axonal marker neurofilament-H [19]. To identify the proteins that interact with KIF17, the authors used the tail domain of KIF17 as bait in yeast-2-hybrid screen and found it bound to the first PDZ domain of the scaffolding protein mLin10 [19]. Using immunoprecipitation with brain lysate and a vesicle fraction, they confirmed this interaction and demonstrated mLin10 forms a complex with mLin2 and mLin7, which in turn binds to the NMDA receptor subunit NR2B. The authors used four other kinesins in the same experiment to show specificity of KIF17 binding to mLin10. Dominant-negative inhibition of KIF17 resulted in a decrease of NR2B at post-synaptic sites and an increase of NR2B-positive puncta in the soma. This suggests that KIF17 plays a role in the distribution of NR2B, but its exact role remains unclear.

To study transport of KIF17, Guillard et al. transfected YFP-KIF17 into neurons and found it labeled vesicles that moved in the axon and dendrites. They measured 30 moving vesicles and found the average velocity to be

0.78um/sec. This is the first time transport of KIF17 labeled vesicles was documented. However, it must be noted that vesicle labeling by KIF17 does not equate to transport by KIF17. This result indicates they are capable of live-cell imaging but they did not try a two-color assay with KIF17 and NR2B. Two-color imaging with a cargo, although difficult, would be strong evidence that they are moving on the same vesicle.

In a different assay, Song et al. used a chimera kinesin approach to provide conclusive evidence for interaction for KIF17 and NR2B [47]. In this assay, the motor domain from one kinesin is attached to the tail domain from another kinesin. The output is the mis-localization of the cargo by the chimera kinesin. This technique works especially well with dendritic cargos since mis-localization of a dendritic cargo to the axon may be simple to detect. Using a $KIF5B_{motor}$ -17_{tail} chimera, they show expressed NR2B is mis-localized to the axon strongly confirming this interaction.

A dominant negative screen was used by Don Arnold's group to identify kinesins that bind to a specific dendritic cargo [48]. This strategy uses an expressed motor-less kinesin that is assumed to dimerize with the endogenous kinesin and disrupt the ability of the motor to transport its cargo. In theory, it is reasonable that if this kinesin were transporting this cargo, then disrupting the kinesin would alter the cargo's trajectory to its destination. To identify the kinesins binding to the dendritic potassium channel Kv4.2, the authors screened KIF21B, KIF5B, KIF5A, KIF17 and KIFC2 tails because each are implicated in dendritic transport or thought to be localized to the dendrites. Of all of the tails

tested, only dominant-negative KIF17 affected the localization of Kv4.2 by blocking its localization of Kv4.2 to the dendrites. In this paper, the images are convincing in that Kv4.2 is not localized to the dendrites when co-expressed with KIF17's tail. As a control to test whether total protein packaging and transport was functional, they looked at the co-localization of another protein, CD8, and found it distributed normally to the axon and dendrites. However, if KIF17 works in the dendrites, the distribution of another dendritic protein, preferably one not already associated with KIF17, would act as a control for dendrite specific proteins. Furthermore, the authors claim that transport of Kv4.2 was disrupted, yet they do not show any transport of KIF17 vesicles.

Another kinesin that has been implicated in transport of dendritic cargos is the KHC isoform KIF5B. Setou et al. used KHC as bait in a yeast-2-hybrid and identified GluR2 Interacting Protein 1 (GRIP1) as a binding partner. This interaction was confirmed in an immunoprecipitation assay, using several other kinesin tails for negative controls to test for specificity. In one IP, they provide an input lane but do not report the percentage of input used for the analysis. The efficiency of the interaction is therefore uncertain. Fibroblasts cultured from KIF5B knockout mice show GRIP localized near the center of the soma whereas wild-type mouse fibroblasts have GRIP1 at the cell periphery. Transfection of KIF5 rescued the localization of GRIP1 to the periphery in knockout fibroblasts, though why this was not shown in neurons is unclear. In wild-type fibroblasts, KIF5B tail was used in a dominant negative assay and was shown to disrupt GRIP1 localization. This confirmed a result in the aforementioned paper from the

Arnold group that also reported that dominant negative KIF5B inhibited localization of GluR2 to the dendrites. Taken together, both examples implicate KIF5B interacting with GRIP1, but neither report actual transport data to support the hypothesis that KIF5B transports GRIP1.

The story of KIF1A provides more evidence that a kinesin interacts with dendritic cargo. KIF1A, a member of the Kinesin-3 family, was shown to associate with Liprin-α, a scaffolding protein for AMPA receptors in dendrites[49]. Shin et al. performed a Y2H using liprin-α as bait and identified a region of KIF1A that interacted with it. In this example, using cargo as bait rules out the need for controlling for other kinesins interacting under these conditions, but does not rule out that other kinesins can bind to liprin-α. In a follow-up immunoprecipitation experiment using light membrane enriched fractions and synaptic vesicles of rat brain they report KIF1A interacted with liprin-α GRIP, GluR2/3, synaptotagmin, RIM and beta-pix. All of these proteins can be found in the post-synaptic compartment and the authors propose these proteins exist in a complex. In the same experiment liprin-α immuno-precipitated KIF1A, GRIP and RIM (RAB3 interacting protein). Yet compared to the amount of input, very little KIF1A was immuno-precipitated. The authors did not test for other kinesins binding to these proteins in these conditions, thus it is unclear if the interaction is specific for KIF1A. They further propose the interactions provide evidence that this complex is transported by KIF1A but they do not present evidence to support this. Ideal evidence for this would be two-color transport assay of the various combinations

of proteins. Overall there is evidence of interaction but no demonstration of KIF1A transporting liprin-α associated vesicles.

Two other kinesins have been reported as dendritic kinesins. KIF21B of the Kinesin-4 family and KIFC2 of the Kinesin-14 family were found to immuno-localize to the somatodendritic domain of neurons [50, 51]. KIFC2's motor domain is located at its C-terminus and shares homology to KAR3, a yeast kinesin, known to translocate to the minus ends of microtubules. Because of this it is thought that KIFC2 is minus-end directed motor and thought to be important for dendritic transport because it is less likely to enter the axon. Immunoprecipitation experiments with KIFC2 did not yield any binding partners. KIF21B also does not have putative interacting proteins.

Taken together, evidence exists for different kinesins interacting with dendritic cargos. However, while all examples claimed interaction, none actually demonstrated transport of the cargo by a kinesin. Presently, it seems observing live transport of a kinesin together with its cargo or using a chimera strategy are the best techniques available to provide conclusive evidence that a kinesin can interact with a particular cargo.

Selective transport: Kinesin-Microtubule based mechanisms

In this section I will present data from our lab and others that discuss the models for selective transport involving kinesins. One idea, the smart motor hypothesis, suggests the motor domain of the kinesin is suited to walk on specially modified axonal and dendritic microtubules. The other suggests that a

protein or protein complex on the vesicle can steer the kinesin-vesicle complex to the correct domain.

Smart motor hypothesis

One approach to test the smart motor hypothesis is to determine which of the motor domains of kinesins are able to walk in the axons, dendrites or both. To test this, the tail domain of a kinesin is removed creating a motor domain that is unregulated and constitutively active. A constitutively active motor domain will translocate along microtubules toward the plus end and in neurons this results in an accumulation of the motor domain at the tips of axons and dendrites [23] NH).

There are 3 types of vesicle transport that have been observed live in cultured hippocampal neurons. Vesicles can transport non-selectively to the axon and dendrites (CD8), in the axon and dendrites but with a bias to the axon (NgCAM, BDNF) or selectively in dendrites (TfR, LDLR, Kv2.1, Rab11b, mannose-6-phosphate receptor) [6, 52]. Based on the three observed behaviors of vesicle transport described above, if the motor domain were in control of vesicle transport then one might expect to find motor domains that accumulate in a similar manner as the observed vesicle transport, that is, either non-selectively, biased to the axon, or dendrite selective.

Huang and Banker carried out such experiments with 14 kinesins expressed in hippocampal neurons and thought to mediate vesicle transport [23]. They found only two patterns of accumulation: axon only and non-selective. While no dendrite selective motor was discovered, clearly some motor domains

are more suited to travel on dendritic microtubules than others. Because these motor domains are able to accumulate at dendrite tips, they remain candidates for kinesins that move dendritic cargo.

The motor domains that are able to accumulate at the tips of dendrites in the truncated motor assay are of special interest because their axonal accumulation may be an artifact of the experimental design. Since the motor domains are constitutively active, the axon could act as a sink for the motors because the microtubules in the axon are oriented with their plus-end distal to the cell body. It is plausible that these dendrite-selective candidates transport more efficiently in the dendrites compared to the axon, but the truncated motor assay is not sensitive enough to show this difference. In chapter two of this thesis, I test this hypothesis with KIF13B from the Kinesin-3 family.

The microtubule motor interaction is important since it has been shown that manipulating this interaction can change the selectivity of the motor domain. For example Huang and Banker showed the switching the MT binding loops between KIF1A and KIF5C allowed the normally axon selective KIF5C to accumulate in the dendrites. Reed et al. showed that pharmacologically increasing tubulin acetylation increased the number of neurite tips that co-stained for JIP1, which is transported by Kinesin-1 [53]. Furthermore, kinesin heavy chain moved WT microtubules in a microtubule gliding assay faster than microtubules that had been mutated to disallow tubulin acetylation [53]. In developing neurons KIF5C will accumulate at the tip of the axon [54]. However, globally increasing microtubule post-translational modifications with taxol

decreased the axon selectivity of KIF5C and redistributed it to other neurites in a young neuron [55]. Taken together, these results indicate that the microtubule-motor domain interaction is important, but do not elucidate exactly how the motor domain contributes to selective transport.

Song et al. hypothesized that certain motor domains are better suited to traffic through the axon initial segment (AIS) and that this is one explanation for selective transport [47]. To demonstrate whether a proposed dendritic motor and axonal motor translocate differentially in the AIS, the authors use FRAP experiments with constitutively active GFP-KIF5B and GFP-KIF17. In these experiments, the authors photobleached a region within the AIS and measured the rate at which non-bleached, fluorescently-labeled motor domains returned to the region. The authors observed that the signal from KIF5B is able to recover quicker than KIF17. They reason it is because KIF5B is more efficient than KIF17 at axonal transport. In both cases, I would expect the anterograde to recover much quicker than the retrograde due to the microtubule orientation. The anterograde signal consists of both actively transporting motors on microtubules and inactive motors moving by diffusion, whereas the retrograde signal is dependent only upon diffusion of the fluorescently labeled motor domain that is not attached to the microtubules. Fluorescent signal from the KIF5B motor domain recovers at nearly the same rate from both the anterograde and retrograde direction suggesting diffusion is the driving force behind the recovery. The same is true for KIF17 motor domain. The results of these experiments do not directly support their hypothesis that KIF17 is a weak motor.

As previously discussed, Song et al. also used chimera to show an interaction between a kinesin tail and a candidate cargo [47]. These experiments further support the smart motor theory for selective transport since they demonstrate the chimeras redistribute their cargo based on the hypothesized region of the cell where the motor domain prefers to transport. The authors demonstrate a $KIF17_{motor}$-$5B_{tail}$ chimera restricts expressed GFP-VAMP2 (synaptobrevin) from the axon. Typically synaptobrevin is present throughout the axon and dendrites, but is only inserted and retained in the axonal membrane [56]. This result suggests that the tail of KIF5B binds to VAMP2 vesicles and the KIF17 motor domain may not be able to transport this vesicle in the axon. Alternatively, it is possible this chimera affects the selective retention in the axonal membrane. Since transport of these vesicles was not shown, it is difficult to know if the vesicles enter the axon or not. Similarly, this chimera may somehow disrupt VAMP2 packaging into its normal axonal vesicle. Neither scenario is addressed in the paper. Nonetheless, based on these results, the authors conclude that KIF17 is a weak motor unable to carry the KIF5C cargo (VAMP2-positive vesicles) through the axon initial segment. The authors offer no proof, however, that these chimera kinesins actually transport vesicles. Furthermore, the weak motor (KIF17) hypothesis does not work for KIF5B. It is suggested to transport both an axonal (JIP) and dendritic (GRIP1) cargo [36, 37]. The authors do not discuss how KIF5B is strong enough to enter the axon when it transports JIP, but becomes weak when transporting GluR2 vesicles. A control

for other KIF5B cargoes (JNK or GRIP1) would help to show if all KIF5B cargoes are now retained in the dendrites.

The chimera results are interesting and strongly support the concept that tails of the kinesins can bind to these particular cargos within a neuron. Characterizing the transport behavior of these vesicles may be useful to establish how efficient a motor domain transports in the axon or dendrites and further if the cargo can influence this transport. Additionally, these chimera provide evidence for the smart motor hypothesis though more experiments are needed to conclusively implicate KIF17's motor domain as a mechanism for selective transport of its vesicle.

Taken together, it is clear the motor domain-microtubule interaction can play a role in affecting kinesin transport but is not sufficient to direct dendrite selective transport. These results partly support a smart motor since the motor domain can recognize differences between different microtubules, but suggest another mechanism is involved for selective targeting.

Cargo steering theory

The second theory involving kinesins and selective transport loosely postulates that the cargo/vesicle steers the kinesin-cargo complex. KIF5 and KIF1A have been shown to interact with axonal as well as with dendritic cargoes, so it seems logical a vesicle's influence over cargo destination should not be overlooked [5, 36, 37, 49]. KIF1A was found in both the pre and postsynaptic compartments [49]. How is it that a kinesin can carry one cargo to a certain

domain but carry a different cargo to a different domain? This is still an open question in the field of selective transport.

Many different interactions between cargo and kinesins have been reported, but very few touch upon the topic of selective transport. Despite the lack of evidence for a mechanism to explain the cargo-steering hypothesis, it is reasonable to consider since the motor domain-microtubule interaction cannot sufficiently explain selective transport. Chapter three of this thesis presents a new method to identify kinesin-cargo interactions within a neuron to further investigate this question.

Other models of dendrite-selective transport

In addition to models for selective transport involving kinesins that were discussed in the previous section, other models involve dynein and myosin. A model involving dynein states that since active dynein cannot enter the axon based on microtubule orientation, dyneins, not kinesins, drive dendrite-selective transport [57]. A final model involves the molecular motor myosin which walks along actin filaments. In this model, myosins are attached to dendritic vesicles and once the vesicle reach the base of the axon the myosins attach to actin to stop the vesicle from entering further into the axon [58]. Both models are discussed in more detail below.

Dynein model for dendrite selective transport

When proposing a model for dendrite selective transport involving dynein, Kapitein and colleagues suggest the microtubule cytoskeleton is the determining

factor controlling selective dendritic transport. Microtubules in mature dendrites have a mixed polarity so dynein and kinesins can transport in either direction [16]. Dynein has long been a candidate for regulating dendritic transport since it translocates towards the minus-end of microtubules [20]. Given that axonal microtubules are polarized mostly with their plus-ends distal to the cell body, it is difficult to envision dynein actively entering the axon. The authors propose that dynein is responsible for dendrite selective transport solely based on this fact. They hypothesize that if dynein is responsible for dendritic transport, then disrupting dynein may allow dendritic vesicles to enter the axon. Using a dominant negative approach to disrupt dynein function [59] the authors demonstrate dendritic vesicles containing GluR2 enter into the axon. Dynein, through its many interacting proteins, is involved in many cellular processes including Golgi localization and late endosome/lysosome transport [60] . Mis-localization of one dendritic protein does not prove it is a direct effect as global protein packaging, distribution, delivery and endocytosis could be affected. In this model, kinesins are not attached to dendritic vesicles but this is not directly addressed. However, the fact that dendritic vesicles enter the axon when dynein is disrupted suggests that a kinesin is present on these vesicles. If dynein is in control, as the authors suggest, why does a kinesin also need to be present? Furthermore, if both kinesin and dynein are on the vesicle, then which motor is actually transporting the vesicles in dendrites? The authors do not show evidence of either motor transporting a dendritic vesicle, nor do they demonstrate that dynein binds to these dendritic vesicles.

In order to provide evidence for their hypothesis that dynein-mediated transport is restricted from the axon, the authors attach dyneins to peroxisomes as an artificial cargo. In neurons, transport into the axon is restricted, which is expected based on the microtubule orientation. This example does not prove that dyneins are moving any or all dendritic vesicles. It merely demonstrates that an active dynein cannot move an artificial cargo into the axon and this is an expected result. In the same experiment attaching a kinesin motor domain moves peroxisomes to the plus-end of microtubules and in neurons the peroxisomes are transported into the axon. This is an expected result based on the microtubule orientation. Based on these experiments the authors conclude dynein is responsible for dendrite selective transport and kinesins are responsible for axonal transport. However, many papers indicate kinesins are capable of binding to dendritic proteins. In the previous section, the KIF5B-17 chimera should not be able to transport NR2B vesicles into the axon since dynein is supposedly on this vesicle and should restrict its entry into the axon. These results are in direct conflict with the dynein model. The dynein model is reasonable in that it makes sense to have a motor that cannot actively enter the axon be the primary motor for dendrite selective transport. However, the evidence provided is not convincing to suggest that dynein regulates dendrite selective transport.

In the third chapter of my dissertation, I will present a new technique that implicates kinesins are bound to dendritic vesicles which contradicts the model presented above.

Myosin model for dendrite selective transport

In the final model of dendrite selective transport reported here, myosins prevent dendritic vesicles from entering the axon. Lewis et al. propose that myosins, already attached to dendritic vesicles, bind to actin in the axon initial segment and either remove the vesicle from the microtubules tracks or actively transport the vesicle back to the soma [58]. The authors of this paper show that the motor-less myosinVa (dominant negative) expression causes mis-localization of two dendritic proteins, GluR1 and EAAT-3, from the dendrites to the axon. Presumably, this dominant negative myosin attached to the vesicle and without its motor domain is unable to bind actin in the AIS resulting in dendritic vesicles entering the axon. However, the authors do not provide evidence that endogenous or expressed myosins are attached to the vesicles. Furthermore, GFP-dominant negative myosinVa localizes to large unknown compartments in the axon and dendrites and they are not discussed. In RNAi knock down experiments of myosinVa GluR1 was mis-localized from the dendrites to the axon. However, the authors do not provide evidence to show actual knockdown, nor do they provide rescue experiments to support their interpretation that the effects observed are due solely to the loss of myosinVa. Also, with both the RNAi and dominant negative experiments myosin may have other roles in the cell including endocytosis and this may disrupt how GluR1 is packaged into endosomes, making it difficult to conclude the disruption of MyosinVa directly interrupts transport of dendritic vesicles [61, 62]. This model proposes that axonal vesicles either do not have myosinVa on them, or contain myosinVa that

is unable to attach to the actin in the AIS. Data for this is not shown or discussed. Furthermore, transport of these vesicles in or near the AIS is not shown.

Experiments discussed in chapter 3 of my dissertation address this model by using kinesins to transport dendritic vesicles into the axon. Furthermore, this model can be tested by the novel technique presented in chapter 3 and will be discussed in the future directions portion of the discussion.

Currently no model actively demonstrates or describes a mechanism for dendrite selective transport actually using transport. Both models presented are plausible and could be involved in some capacity to regulate dendrite selective transport but on their own each paper/model cannot definitively prove selective transport is regulated solely by one mechanism involving one class of motors.

KIF13B

While the overall goal of this thesis is to help elucidate how Kinesin-3 family members are involved the mechanism underlying dendrite-selective transport, one main focus of this thesis is KIF13B. KIF13B demonstrated properties which made it a candidate for a dendrite selective kinesin in two different in-vivo assays. But before I delve into the data for its candidacy, I will present some background information.

KIF13B was originally identified in 1997 along with 15 other novel kinesins [63]. KIF13B is a member of the Kinesin-3 family. Similar to other Kinesin-3 family members, it is thought to be a monomer in solution but transport vesicles

as a dimer [64, 65]. KIF13B has three known protein-interacting domains and two phosphorylation sites in its tail region. Listed in order from the N-terminus, these are a forkhead associated domain (FHA), a MAGUK binding domain (MBS), phosphorylation sites for PAR1B, and a cytoskeletal-associated protein glycine rich domain (CAP-Gly) at its far C-terminus. In this last section of this introduction, I will review some key papers which investigated the cellular role of KIF13B and its interacting proteins.

The first known interacting protein of KIF13B was found in 2000 by Hanada et al. while investigating the role of phosphorylated human discs large (hDlg) in T-lymphocytes [35]. Human discs large is a member of the membrane associated guanylate kinase (MAGUK) family whose mammalian counterparts include PSD93 and 95, SAP97 and 102. PSD-95 and SAP97 are scaffolding proteins in the post-synaptic compartment and are suggested to regulate AMPA receptor trafficking near dendritic spines [66, 67]. Using GST-hDLg delta C-terminus, they pulled down a heavily phosphorylated band of 250 kDa from lysate prepared from the Jurkat 77 lymphocyte cell line [35]. This protein band was identified to be a member of the kinesin superfamily, and the authors originally named it guanylate-associated kinesin or GAKIN, otherwise known as KIF13B. Characterization of KIF13B using northern blot analysis revealed its mRNA was prominent in the brain, kidney and pancreas, as well as in Jurkat cells. Deletion constructs of KIF13B used in immunoprecipitation experiments revealed it binds to hDLg in a region located within the stalk of KIF13B (607-831),

termed the Maguk Binding Stalk or MBS [68]. This region of KIF13B was shown to interact with the GUK domain of hDlg, as well as the GUK domain of PSD95.

This same group later characterized the activity of KIF13B. In a microtubule-stimulated ATPase activity assay, Yamada et al. tested the ATPase rate of the kinesin. Interestingly, the authors found that full-length KIF13B is inactive, but a truncation of KIF13B that included the motor and FHA domains was active [69]. This led the authors to conclude that full-length KIF13B was regulated intra-molecularly, similar to KIF5, KIF1A and KIF17 [70-72]. To test this hypothesis, Yamada and colleagues assayed for regions of KIF13B that were able to bind to the motor domain in biochemical pull-down assays, and found that a region in the stalk domain (607-989) interacted with the motor domain. Surprisingly, this region also interacts with the GUK domain of hDLg. The authors next investigated whether binding of hDlg could activate KIF13B in this microtubule-stimulated ATPase activity assay. Full-length hDlg could not activate KIF13B, presumably due to its own intra-molecular interactions [73]. Multiple deletion constructs of hDlg were then incubated with full-length KIF13B to test whether a region of hDlg could activate KIF13B. One truncation of hDlg, consisting of the SH3 and GUK domains, was able to stimulate KIF13B ATPase activity. Surprisingly, the GUK domain of PSD95, while able to bind to KIF13B, did not stimulate KIF13B ATPase activity. It is interesting to note that there are isoforms of hDlg which have insertion cassettes (I2, I3 or I5) between the SH3 and GUK domains. Of all the isoforms of SAP97, the SAP97 containing the I3 insertion is more dendritically polarized than the others [66]. SAP97 (I3) has

been shown to modulate AMPA receptor surface expression and bind indirectly to actin through protein 4.2, an actin binding protein.[66]. The SH3-I3-GUK region of hDlg was able to activate KIF13B, but the splice variant SH3-I2-GUK was unable to activate KIF13B. These results strongly suggest hDlg binds to and activates KIF13B. However, full-length hDlg was unable to activate KIF13B presumably due to the closed conformation of hDlg. This observation suggests that another element may 'open' hDlg and activate KIF13B.

In 2005, centaurin-α1 was identified as a novel binding protein for KIF13B. Centaurin-α1 is an ADP ribosylation factor 6 (ARF6) GTP-activating protein (GAP). It comprises an N-terminal zinc finger that contains the ARF-GAP domain, multiple ankyrin repeats, and two C-terminal PH domains [74]. Over-expression of centaurin-α1in hippocampal neurons increases dendritic branching and dendritic filopodia [75]. ARF6 has been implicated in regulating dendritic branching in neuronal cultures, in addition to co-localizing with PSD95 in dendritic spines [76]. Because I will present evidence that KIF13B is a candidate dendrite-selective kinesin, it is important that known interacting proteins have been implicated in dendrite-specific processes.

Centaurin-α1, also known as PIP3 binding protein, was used as bait in a yeast-2-hybrid screen which demonstrated that it interacted with a region of KIF13B (387-649) [77]. The region of KIF13B that binds centaurin-α1 was later determined to contain a forkhead-associated domain (FHA). Forkhead-associated domains are usually found in transcription factors and are known to bind to phosphorylated threonine residues [78]. Using deletion mutants of

centaurin-α1, the authors identified the N-terminal ARF-GAP domain as the minimal region required for binding KIF13B. Using purified proteins in a biochemical pull-down assay, they showed that this interaction is direct and that the minimal region of KIF13B that binds centaurin-α1 is amino acids 455-557 which they labeled as the FHA domain [79]. In HeLa cells, centaurin-α1 lacking its N-terminal KIF13B binding domain failed to localize to the periphery when co-expressed with GFP-KIF13B, whereas wild-type centaurin-α1 did overlap with KIF13B at the periphery. Further evidence for an interaction was provided when a crystal structure of the FHA domain and centaurin-α1 was solved and provides more conclusive evidence of binding [80].

Chishti's group continued the investigation between the interaction of KIF13B and centaurin-α1 [79]. In this paper the authors performed two *in vitro* transport assays. First, a microtubule gliding assay showed KIF13B was a plus-end motor and moved microtubules at an average velocity of 1.66 um/sec. Second, using a PIP3 liposome motility assay, the authors demonstrate that KIF13B can carry PIP3 liposomes in the presence of centaurin-a1 at an average rate of 0.7 um/sec. As a control liposomes with PIP2 were not transported by KIF13B. These results suggest centaurin-α1 can serve as an adaptor protein to link KIF13B to PIP3-containing vesicles. Together, the biochemistry and in vitro transport data support the interaction between centaurin-α1 and the FHA domain of KIF13B and suggest centaurin-α1 can act as a link to vesicles.

Several other papers have been published concerning the motility and phosphorylation of KIF13B in other cell types. Huckaba et al. determined that

KIF13B is predominantly a monomer in solution but is able to move processively as a dimer [81]. In an in-vitro single-molecule motility assay using the KIF13B motor domain with varying lengths of its tail domain, the authors showed different lengths of KIF13B were found to have increased bouts of activity suggesting that KIF13B moves differently depending on the length of the tail present in the assay. They also demonstrated two-color live cell imaging in insect cells that strongly suggests that KIF13B associates with RAB5-positive endosomes. Yoshimura et al. demonstrated that Par1b can phosphorylate KIF13B and tried to relate this phosphorylation event to axonal outgrowth [82]. The biochemistry data are convincing that Par1B phosphorylates KIF13B but the *in vivo* data to support a functional role of this KIF13B phosphorylation state are inconclusive. KIF13B was found to be expressed in Schwann cells and in a complex with hDlg, sec8 and MTMR2 near the cell surface [83]. No transport of KIF13B was reported and interaction of KIF13B with this complex was suggested based on immunostaining and immunoprecipitation. In immune cells, KIF13B was shown to interact with CARD11 – a signaling scaffold for IKK, TRAF and BcL10 located at the immune synapse of activated lymphocytes [84].

This thesis began with an observation I made shortly after joining the Banker lab. I found that full-length GFP-KIF13B labeled vesicles that do not enter the axon. This is the only kinesin to date that labels dendritically polarized vesicles. The first data chapter of my dissertation investigates the hypothesis that intrinsic properties of the KIF13B motor itself are responsible for dendrite-selective transport. Ongoing work in the lab showed that a constitutively-active

KIF13B motor domain accumulated in both axons and dendrites. While at a first glance this result appears to contradict my hypothesis, an alternative interpretation of this result gave me reason to further investigate the ability of the motor domain to transport in the axons and dendrites. I utilized a new cell-based assay to compare the efficiency at which the motor domain transported an artificial cargo in the axon and dendrites. My results showed the motor domain of KIF13B cannot account for dendritic selectivity on its own. I also confirmed a regulatory mechanism for KIF13B which may provide the basis for future investigations into the role of KIF13B regulation in dendrite selective transport. Since the motor domain was not sufficient for regulating selective transport, it seemed reasonable to next investigate if the cargo contributed to transport selectivity.. Determining the cargo of KIF13B is a first step toward investigating the cargo's involvement in selective transport and this is the focus of the second data chapter of my dissertation. Along with others in the lab, I developed a new, cell-based assay to identify the kinesins that bind to dendritic cargos. Our results establish that the tail domain of KIF13B is capable of binding to vesicles that contain TfR and LDLR, two well-studied dendritically polarized proteins. This technique can be further utilized to identify other proteins important for the kinesin-vesicle interaction and perhaps give insight into a mechanism for dendrite-selective transport.

Chapter 2: Investigation of KIF13B-mediated dendrite-selective transport

Introduction

Neurons comprise two distinct cellular domains: somatodendritic and axonal. The division of the neuron into these domains is essential for carrying out its function, which is sending and receiving information. Each domain requires a special complement of proteins to perform its specified function. For example, while both domains require similar housekeeping proteins, different synaptic proteins must be delivered to the pre- and post-synapses in the axon and dendrites, respectively. The neuron achieves compartmentalization of specialized proteins through the process of selective transport. For example, the dendrite-specific proteins transferrin receptor (TfR) and low-density lipoprotein receptor (LDLR), are inserted into the membranes of vesicles and transported primarily in the dendrites. Rarely do they enter the axon, and the mechanism for this preferential localization is a long standing debate.

Neuronal membrane transport is carried out by kinesin and dynein motor proteins on microtubule tracks and by myosin motor proteins on filamentous actin. Dendrite-selective vesicle transport has been demonstrated in early stages of neuronal development when the microtubules in the neurites are oriented with their plus-ends distal [85]. Since, neither dynein nor myosins can be responsible for this observed transport, our research focuses on kinesin motor proteins [86]. The overall goal of this chapter is to investigate the mechanism of kinesin-mediated dendrite-selective transport.

Identifying kinesins that move dendritic vesicles is a first step toward understanding dendrite-selective transport. One approach is to express GFP-fused kinesins in neurons and test whether the kinesins bind vesicles moving only in dendrites. Our lab tested this strategy with Kinesin-1 and Kinesin-4 families, but they failed to bind to vesicles. However, Lee et al. showed that KIF1A of the kinesin-3 family successfully attaches to vesicles that moved in the axon and dendrites [87]. In the current study, I tested three other members of the kinesin-3 family using this assay. GFP-KIF1Bβ and GFP-KIF13A both bound to vesicles that moved in the axon and dendrites. Interestingly, GFP-KIF13B bound to vesicles that trafficked almost exclusively in the dendrites. This result identifies KIF13B as a candidate dendrite-selective kinesin.

Our lab tested a different method to identify dendrite-selective kinesins that did not result in KIF13B as a candidate [23]. This method studied the ability of the motor domain alone to move in either the axon or dendrites. Deleting the kinesin's tail region creates a truncated, constitutively-active motor domain. These motor domains translocate on microtubules and accumulate at their plus-ends. In neurons, this results in kinesin motor domains accumulating at the tips of axons and dendrites. If a dendritic kinesin existed, then expressing the motor domain alone may result in selective accumulation at the tips of dendritic microtubules. Huang and Banker expressed the motor domain of KIF13B and found instead that it accumulated at the tips of both axons and dendrites.

Neither assay expressing full-length GFP-labeled kinesins or the truncated motor domains, definitively proves the existence of a dendritic kinesin. Rather,

the results point toward candidates for a dendrite-selective kinesin. Is it possible that despite the truncated motor assay result, KIF13B is a dendrite-selective kinesin: can the two results be reconciled? One possibility is that the non-selective behavior exhibited by KIF13B's motor domain in the truncated motor assay is a result of the axonal microtubule orientation. Axonal microtubules are polarized with 90% plus-end distal to the cell body [16]. A constitutively active motor domain may translocate on the 'one-way street', unable to actively return or diffuse back to the cell body. It is conceivable that the KIF13B motor domain travels more efficiently on dendritic compared to axonal microtubules, but the truncated motor assay is not sensitive enough to demonstrate this. It is also possible that the motor domain is not responsible for selective transport, and that another region within KIF13B works alone or in conjunction with the motor to direct dendrite-selective transport.

To test the hypothesis that KIF13B directs dendrite-selective transport, I devised a set of experiments that utilized an artificial cargo assay. This enabled me to test both previous kinesin selectivity assays. If full-length KIF13B has intrinsic dendrite-selective properties, then it should direct selective transport of an artificial cargo. Furthermore, the use of an artificial cargo tests the truncated motor results by comparing the motor domain's ability to transport an artificial cargo in the axon and dendrites.

KIF13B was engineered to acutely link with an artificial cargo: peroxisomes. Full-length KIF13B bound to but was unable to move peroxisomes. The motor domain of KIF13B transported peroxisomes but did not

transport them differentially in the axon compared to dendrites. Various truncation and deletion constructs were created to test regions of KIF13B that allowed peroxisome transport but inhibited axonal entry. Some constructs moved peroxisomes but did not restrict transport to the dendrites. Other constructs were unable to be evaluated since few transport events were recorded. Consistent with a previous report [69], the MAGUK binding stalk (MBS) within KIF13B partially participates in regulating motor activity and peroxisome transport.

Together, the results of these experiments suggest the motor domain up to the second coiled-coil domain does not harbor dendrite selective properties. If KIF13B is dendritic, then the regions beyond the second coiled-coil domain must contribute to KIF13B's localization. Constructs testing these regions failed to give interpretable results in the peroxisome artificial cargo assay.

Results

Identifying candidate kinesins responsible for dendrite selective transport

To identify candidate kinesins that might be responsible for dendrite selective transport, GFP-tagged full-length kinesins were expressed in hippocampal neurons. This strategy has been previously applied to Kinesin-1 and Kinesin-4 family members and none were found to label vesicles (unpublished Banker lab data). Instead, these GFP-kinesins exhibited a diffuse distribution within the neuron resembling soluble cytosolic GFP expression. However, this strategy did work with GFP-KIF1A, as it labeled vesicles that moved bi-directionally in the axon and dendrites [87]. Because this strategy was successful with GFP-KIF1A, I applied it to other Kinesin-3 family members: KIF1Bβ, KIF13A and KIF13B.

These four full-length Kinesin-3 family members were fused to GFP on their N-terminal motor domain, where the fluorophore does not interfere with motor domain activity [23]. 7 to 12 days in-vitro (DIV) neurons were transfected with various GFP-kinesins and allowed to express overnight. The following day cells were imaged live on a fluorescence microscope (Fig 1). Similar to the Kinesin-1 and Kinesin-4 families there was a significant soluble pool for all GFP-kinesins (Figure 1). In some cells, vesicles were apparent, but in most, no vesicle labeling could be found. Low magnification images of representative neurons with labeled vesicles are shown for each kinesin on the left column of

Figure 1. High magnification images of the axon and one dendrite, adjacent to the kymographs, show the vesicles observed during movie acquisition.

Time-lapse movies of individual axons and dendrites were transformed into kymographs to analyze vesicular transport. In kymographs, diagonal lines represent transport events, with positive slope indicating transport away from the cell body (anterograde) and a negative slope indicating transport toward the cell body (retrograde). Horizontal lines indicate vesicles that did not move during the movie. Kymographs are oriented such that time is along the x-axis and distance is on the y-axis; therefore, the slope of the line also indicates the velocity in microns per second (µm/sec).

GFP-KIF1A, GFP-KIF1Bβ and GFP-KIF13A visibly labeled vesicles that move in both the axon and dendrites (Fig. 1 A, B, C). GFP-KIF1A (two of three cells, [5, 87]) and GFP-KIF1Bβ (five of five cells) labeled vesicles that move anterogradely and retrogradely in the axon and dendrites (Fig 1 A and B). Vesicles labeled with both KIF1A and KIF1Bβ, moved longer distances in the axon than in the dendrites. KIF13A-labeled vesicles moved predominantly in the anterograde direction in the axon while vesicles in the dendrites traveled shorter distances and at slower speed as measured by the positively-sloped lines on the kymograph (8 out of 8 cells, Fig. 1C).

In contrast, in 16 of 20 cells analyzed, GFP-KIF13B-labeled vesicles were predominantly found in the dendrites with few or no vesicles that entered the axon (Fig 1D). Interestingly, 13 of the 20 cells analyzed GFP-KIF13B-labeled

vesicles moved bi-directionally for long distances in the dendrites (Fig 1D) much different than the dendritic transport from the other Kinesin-3 family members.

Testing the ability of full-length KIF13B to direct dendrite transport by chemically linking it to an artificial cargo.

KIF13B is unique because it was the only Kinesin-3 member found to have a dendrite selective distribution. If KIF13B has intrinsic properties for selective transport, then it should transport any vesicle population exclusively in dendrites. To test this, an artificial cargo, peroxisomes, was linked to GFP-KIF13B. Peroxisomes are found in abundance in cells and are primarily responsible for catabolism of long-chain fatty acids. They were used because they can be brightly labeled for visualization and are mostly motionless in the cell body. The increased signal-to-noise ratio and reduced motion artifacts reduce experimental variability compared to GFP-Kinesin-3 imaging.

KIF13B was linked to peroxisomes using an inducible protein-dimerization kit [Ariad pharmaceuticals (Clontech) [88]]. This method is based on two protein domains found endogenously, the FK506 binding protein (FKBP) and the FKBP12 rapamycin binding (FRB) protein, which can be linked together by rapamycin. Fusion of these domains onto proteins of interest enables them to be linked by adding the rapamycin analog AP21967. In these experiments, FKBP was fused to the C-terminus of GFP-KIF13B. Peroxisomal biogenesis factor 3 (PEX3) had mRFP and FRB fused to its C-terminus. PEX3 is an integral

peroxisome membrane protein involved in peroxisome biogenesis and is found in nearly all peroxisomes [89, 90].

Neurons aged 7-12 DIV were co-transfected with full-length GFP-KIF13B-FKBP and PEX3-mRFP-FRB. The following day transfected neurons were imaged live on a fluorescence microscope. Prior to addition of the linker drug, peroxisomes were largely stationary and located in the somatodendritic domain (Fig 2C). GFP-KIF13B-FKBP signal was diffuse and few dim vesicles could be observed (Fig 2A). This result was similar to neurons expressing the full-length GFP-KIF13B (Fig 1D). Neurons were imaged at 10-15 minute intervals after linker drug addition to monitor peroxisome transport. GFP-KIF13B-FKBP began to accumulate onto peroxisomes 15-20 minutes after drug addition; this accumulation was apparent because the red peroxisomes gained green fluorescence (Fig 2F). Surprisingly, attaching KIF13B to peroxisomes did not result in the movement of peroxisomes. Kymographs of peroxisome transport revealed no change in transport after adding the linker drug (Fig 2H).

The motor domain of KIF13B transports peroxisomes efficiently in axons but not in dendrites of mature neurons

Full-length KIF13B FKBP can be targeted to peroxisomes but is unable to move them. It is possible that this kinesin is regulated by inter and intra-molecular interactions. Domains within a kinesin can interact intra-molecularly and inactivate it [15, 71]. Alternatively, the kinesin could be regulated by soluble proteins or proteins related to its endogenous vesicle that are required for cargo

transport. A full-length kinesin attached to a peroxisome, perhaps un-naturally, may affect its ability to interact intra or inter-molecularly. To address this issue I removed the tail domain of KIF13B. This type of truncation creates a constitutively active motor domain [54]. Truncated KIF13B has been shown to accumulate at the tips of axons and dendrites when expressed in cultured hippocampal neurons [23]. This demonstrates that they are functional motors and constitutively active. Kapitein and his colleagues have shown that a constitutively active, truncated KIF5C is able to move peroxisomes using this dimerization strategy [57]. Use of the KIF13B motor domain alone tests whether the motor domain has intrinsic properties to direct dendrite-selective transport.

To test if the motor domain of KIF13B transports peroxisomes more efficiently in the dendrites compared to the axon, KIF13B's motor domain and the first coiled-coil domain (1-393) were fused to GFP and FKBP on the N and C-terminus respectively. Previous reports found the KIF13B motor domain does not spontaneously dimerize and must be forced dimerized to accumulate at dendritic tips [23]. Based on these results, the KIF13B motor domain was engineered with a homo-dimerization domain (Fv) upstream of the FKBP to create motor domain dimers and hetero-dimerization domain to link the peroxisomes. Fifteen to thirty minutes prior to imaging, the linker drug (hetero-dimerizer) was added to the neurons to link the motor onto peroxisomes. Neurons expressing GFP-KIF13B (393) Fv-FKBP and PEX3-mRFP-FRB were recorded live before homo-dimerizer addition and in 10-15 minute intervals after homo-dimerizer addition. Prior to adding the homo-dimerizer drug the

peroxisomes mostly occupied the somato-dendritic region (Fig. 3C) and were relatively motionless (Fig. 3E), while the GFP-motor domain had a diffuse distribution and filled the cell (Fig 3A). Ten to fifteen minutes after adding the homo-dimerizer drug, numerous anterograde transport events were observed in the axons of mature stage 4 neurons. The transport of axonal peroxisomes was processive – that is, they traveled for many microns without stopping or changing direction. Surprisingly the dendritic transport was significantly less processive compared to the axon (Fig. 3F). This indicates the motor domain does not have an inherent preference for translocating on dendritic microtubules. In fact, the motor domain of KIF13B transported better on axonal microtubules than dendritic.

KIF13B motor domain transports peroxisomes, an artificial cargo, in young neurons

Peroxisome transport events in stage 4 dendrites traveled short distances and frequently changed direction and thus, are difficult to interpret since the events are different from other dendritic transport observed previously. In comparison, vesicles with either full-length GFP-KIF13B vesicles (Fig 1D), TfR and LDLR (data not shown) traveled longer distances and did not change direction as frequently. In mature dendrites the microtubules are oriented 50% plus-end distal and 50% minus-end distal. Thus, a peroxisome with many constitutively active motor domains may be traveling on one or more adjacent microtubules and pulled in opposite directions simultaneously. This could explain

the short events with frequent changes that were observed in the peroxisome assay.

To test this, younger, stage-3 neurons were used. In these cells the microtubule orientation in dendrites is similar to the axon; 90% plus-end distal to the cell body [85]. DIV0 neurons were co-electroporated with GFP-KIF13B (1-393)-Fv-FKBP and PEX3-mRFP-FRB. One to two days post-electroporation, neurons were imaged live on a fluorescence microscope. Similar to previous experiments, time-lapse movies were taken before and in 10 to 15 minute intervals after drug addition. As in stage 4 neurons, before addition of the linker drug peroxisomes were non-motile (Fig. 4D). Compared to stage 4 neurons, peroxisomes appeared even more localized to the cell body and rarely appeared in proximal dendrites (Fig. 4B). Again, the motor domain was diffuse and filled the neuron (Fig. 4A). After adding the linker drug, the KIF13B motor domain attached to peroxisomes, which was confirmed by the appearance of green peroxisomes. Twenty minutes post drug treatment, peroxisomes were transported efficiently out of the soma and into the axon and dendrites of stage 3 neurons (Fig 4E). As expected, the peroxisomes traveled almost exclusively away from the soma, indicating the uniform microtubule orientation. Ten to fifteen minutes after initial transport peroxisomes and the associated motor were found at the tips of the axon and dendrites (Data not shown).

This experiment demonstrated KIF13B motor domain transported peroxisomes with nearly the same velocity and processivity in the axon and dendrites (Table 1). Based on these measurements, we surmised that the motor

domain did not have a preference for dendritic microtubules over axonal microtubules. Furthermore, since the transport in stage 3 dendrites is more processive, it seems likely that the mixed microtubule orientation was responsible for the short events and frequent changes in direction that were observed in stage 4 (Fig. 3). Because dendrite transport in stage 3 is more easily interpretable than in stage 4, future experiments will be carried out in stage 3 neurons.

Regions of KIF13B do not confer dendrite selective transport properties to the motor domain

Throughout the course of these experiments it became clear the motor domain was able to transport peroxisomes without the addition of the homo-dimerizer. Peroxisomes transported with and without the homo-dimerization drug moved at similar velocities and processivities. For this reason, in the following experiments all constructs were made without the homo-dimerization domain.

Since the motor domain did not move peroxisomes selectively (Fig. 3) and the full length KIF13B-labeled vesicles were dendritic (Fig. 1), it is possible the tail domain is involved in regulating activity and perhaps selective transport. The tail domain contains several known protein-interacting domains. Downstream of the motor domain lies an FHA domain (423-557aa) that is known to interact with centaurin-α1, a PIP3 binding protein [79]. After the FHA domain resides the second coiled-coil domain (CC) of KIF13B (607-648). Coiled-coil domains commonly mediate protein-protein interactions and in kinesins often act as

dimerization domains [65]. Downstream of the second coiled-coil domain is a region housing the Maguk Binding Stalk (MBS) (607-989aa). This region binds to a domain of SAP97 – a post-synaptic scaffolding protein and interacts with the motor domain [69]. The remaining region of the C-terminus houses a PAR1B phosphorylation site and a CAP-Gly domain. PAR1b kinase is involved in cell polarity and its phosphorylation of KIF13B was shown to inhibit axon formation [82]. CAP-Gly domains are known to bind tyrosinated tubulin and are found in proteins involved with regulating microtubule organization and dynamics [91, 92]. However, in the case of the CAP-Gly domain of KIF13B it has not been shown to play a role in binding tubulin.

To test whether specific regions within the tail were responsible for dendrite selective transport or regulation of KIF13B, several KIF13B truncation and deletion mutants were created. Freshly dissociated hippocampal neurons were co-electroporated with the various KIF13B constructs and the peroxisome marker. One to two days later, the electroporated neurons were imaged on a fluorescence microscope. KIF13B constructs focusing on each region of the tail were tested using the peroxisome assay and the results of these experiments are shown in Figure 5.

All tested constructs transported peroxisomes, but none confined peroxisomes to dendrites. However, for each construct tested, different velocities and processivities were observed. KIF13B constructs comprising amino acids 1-568aa and 1-648aa transported peroxisomes faster than the motor domain alone (table1). KIF13B 1-990aa, which included the MBS, transported peroxisomes but

not nearly as processively or robustly as the previous two constructs. Few long-range events (>3um) were recorded over many experiments, yet the peroxisomes always reached the axon and dendrite tips (data not shown). KIF13B Δ393-1282 transported peroxisomes to the tips but again few peroxisome transport events were recorded. Since these last two constructs were unable to be observed transporting peroxisomes, no conclusion can be drawn for their selectivity between axons and dendrites.

The MBS contributes to the regulation of the full-length KIF13B

To investigate whether the MBS region contributed to regulating KIF13B-mediated peroxisome transport, this region was deleted from GFP-full-length KIF13B-FKBP. Previous reports showed the MBS region is able to interact with the motor domain of KIF13B and so it was suggested that this interaction may be an auto-inhibitory mechanism [69]. At DIV 0, neurons were co-electroporated with GFP-KIF13B ΔMBS FKBP and PEX3-mRFP-FRB. One to two days later, neurons were imaged live on a fluorescence microscope.

GFP-KIF13B ΔMBS-FKBP transported peroxisomes to the tips of the axon and dendrites. Since the full-length KIF13B (Figure 2) was unable to move peroxisomes, this suggests that the MBS plays an important role in regulating KIF13B-mediated transport. In most cells peroxisomes moved slowly and non-processively (Fig. 6A and B). One possible explanation for this is that KIF13B was carrying peroxisomes in an un-natural position, on the C-terminus,

compared to where it is suggested to carry cargo [68]. Therefore, to mimic a suggested protein binding site, GFP-KIF13B was engineered with the FKBP in place of the MBS. GFP-KIF13B-FKBP-ΔMBS construct transported peroxisomes to the tips of the axon and dendrites. In most cells, the transport was again slow and non-processive but some faster, more processive events could be seen (Figure 6C and D) that were not observed in the previous construct when the FKBP was on the C-terminus.

Both KIF13B constructs lacking the MBS were able to transport peroxisomes better than the full-length wild-type KIF13B. This suggests the MBS can act as an inhibitory domain towards KIF13B transport. However, since the transport of the MBS deletion constructs was never as robust when compared to the motor domain, another region in the C-terminus may contribute to the regulation of KIF13B when carrying peroxisomes.

Comparison of KIF1A monomer and dimer in peroxisome transport

Through the course of these experiments we discovered peroxisomes can be transported by the motor domain of KIF13B with and without being homodimerized (Figure 3 and 4). This result is confusing since the motor domain must be dimerized in order to accumulate in the truncated motor assay. To address the discrepancy that the motor domain of KIF13B must be forced-dimerized in the truncated motor assay but not in the peroxisome assay, we tested a known monomeric and dimeric version of KIF1A. KIF1A 1-393aa

includes the motor domain and the first coiled-coil domain. It forms dimers and can spontaneously accumulate in the truncated motor assay[23]. KIF1A 1-360aa includes the motor domain but not the first coiled-coil domain and consequently cannot form dimers, undergo processive movement or accumulate in the truncated motor assay [65, 93] (unpublished data). This construct provides a direct test of whether peroxisomes can be moved by a monomeric motor. Similar to the KIF13B motor domains, an N-terminal GFP and C-terminal FKBP were added to the KIF1A constructs. Freshly dissociated neurons were electroporated with the PEX3-mRFP-FRB and either KIF1A 360 or 393. Movies were taken for each construct before and in 10-15 minute intervals after adding the linker drug. Figure 7 shows kymographs for both of the KIF1A truncation constructs. The monomer is able to move peroxisomes at a rate of 0.6 um/sec while KIF1A 393 transports peroxisomes at a rate of 2.1 um/sec (Table 1).

Discussion

KIF13B's role in selective-dendrite transport

There has been a longstanding debate on the role kinesins play in selective transport. In this chapter, I identified KIF13B as a candidate dendritic kinesin and investigated its dendrite selective transport properties using a peroxisome-based, artificial cargo transport assay. Using this assay I found that although full-length KIF13B attached to peroxisomes, it failed to move them and so the selectivity of the kinesin could not be evaluated. The motor domain was able to transport peroxisomes, but it did not do so selectively, that is - peroxisomes were transported in both the axon and dendrites.

One model of selective transport proposes the motor domain directly regulates selectivity by the efficiency of how it moves on axonal versus dendritic microtubules. For KIF13B this model was disproved, since its motor domain can translocate equally well in both the axon and dendrites (Table 1). If KIF13B is dendritic then a region outside the motor domain may be important for selectivity. To test if the tail domain contributes to selective transport, I created longer constructs to identify regions that may regulate KIF13B motor activity to become selective.

Truncation constructs containing regions up to amino acid 648 were able to transport peroxisomes, but neither selectively nor at different efficiencies in the axon or dendrites. Based on the data presented, regions up to amino acids 648 are not implicated in selective transport. Regions beyond amino acid 648 may

be involved in directing transport, but constructs testing these regions could not be evaluated since few peroxisome transport events were observed.

Regulation of KIF13B activity as a model for selective transport

It is interesting that a region beyond amino acid 648 is known to regulate motor activity. Previous studies demonstrated a KIF13B motor domain construct (1-486) interacted with a region within the MBS (607-989) in a biochemical assay [69]. The authors speculate the binding of the motor to the MBS acts as an auto-inhibitory domain. This is consistent with my data because constructs containing the MBS, KIF13B 1-990 and KIF13B full-length, did not transport peroxisomes efficiently or at all, respectively. Furthermore, deletion of the MBS from full-length KIF13B allows it to transport peroxisomes (Figure 6).

It may be that motor activation and inactivation contribute to the selectivity of KIF13B. In this model, KIF13B is active and allowed to transport vesicles in the dendrites yet once it enters the axon it becomes inactivated. A candidate protein for activating KIF13B is SAP97. SAP97 is a synaptic scaffolding protein that binds to KIF13B within the region of 601-831, near the proposed auto-inhibitory region. In an assay to test motor activity Chishti et al. found that full-length KIF13B was inactive, presumably due to its closed conformation. The authors went on to test and demonstrate a region of SAP97 that activated KIF13B in this assay.

Taken together, these results are consistent with a model for selectivity in which SAP97 binds to and relieves the auto-inhibitory mechanism of KIF13B, allowing vesicle transport in dendrites. Once this complex nears the axon initial segment this interaction is disrupted and KIF13B becomes auto-inhibited. Further experiments to test this model are discussed in chapter 4 of this thesis.

KIF13B dimerization and its ability to transport an artificial cargo

Over the course of these experiments, it became apparent that this artificial cargo assay may not have been working as we had thought. We were unclear if the peroxisomes were transported by kinesin monomers or dimers. It was puzzling that the motor domain of KIF13B must be forced to dimerize in order to accumulate at neurite tips in the truncated motor assay [23], but the motor domain does not need to be dimerized to transport peroxisomes, as discussed briefly in Figure 3.

To test if a monomeric motor can move peroxisomes, we used the motor domain of another Kinesin-3 family member, KIF1A. A KIF1A version missing the first coiled coil domain (1-360aa), which renders it a monomer, allowed us to compare peroxisome transport with a dimeric KIF1A (1-393aa). Interestingly, the monomeric KIF1A (360aa) can transport peroxisomes and does so at a velocity of 0.6 um/sec compared with a rate of 2.1 um/sec for the dimer (Fig 7). Since the KIF1A monomer can transport peroxisomes, it is likely that the motor domain of KIF13B may be doing the same. As a dimer, KIF1A transported peroxisomes faster. In my experiments, I found that longer constructs of KIF13B moved

peroxisomes at a faster rate (table 1). Perhaps this is because they form dimers more readily. A study of the drosophila homolog of KIF13B found that the motor domain does not readily form dimer, but longer constructs shifted the monomer-dimer equilibrium toward dimer [81].

The reason that monomeric kinesins are capable of transporting peroxisomes may be because many copies are bound to the peroxisome. Similar to a microtubule gliding assay, I can envision a scenario where a large number of monomers attached to a peroxisome can move along the microtubules. A single monomer going through its stepping cycle would fall off the microtubule, but multiple monomers hydrolyzing and stepping at different times could keep the peroxisome on the microtubule and move it. To investigate this further, it might be possible to reduce the number of kinesins binding to the peroxisomes, by competing the PEX3-RFP- FRB with a PEX3-GFP containing no chemical dimerization domain. Transfecting neurons with varying amounts of the two constructs and titrating this ratio to a point where the monomer cannot move peroxisomes but the dimer can might allow for a more physiological model of vesicle transport. In addition, binding fewer dimers of KIF13B to the peroxisomes may allow for better transport in the dendrites, since it could reduce the chance of simultaneous binding to microtubules of opposite polarity, which was an experimental flaw. Finally, if KIF13B is normally inactivated near the axon initial segment, binding too many kinesins may reduce susceptibility to this mechanism. It may be that KIF13B does not normally transport efficiently in the axon but this

property was masked because too many kinesins were present on the peroxisome.

The proposed model for KIF13B-mediated dendrite-selective transport suggests inactivation of the kinesin as the mechanism for confining its vesicles to the dendrites. This peroxisome assay tests the specific role for KIF13B and soluble interactor proteins but cannot address the contribution of proteins associated with KIF13B's endogenous cargo. It may be that components of the endogenous vesicle and its associated proteins contribute to making KIF13B transport selective. To study this further requires knowing the endogenous cargo of KIF13B, the topic of the next section of my dissertation.

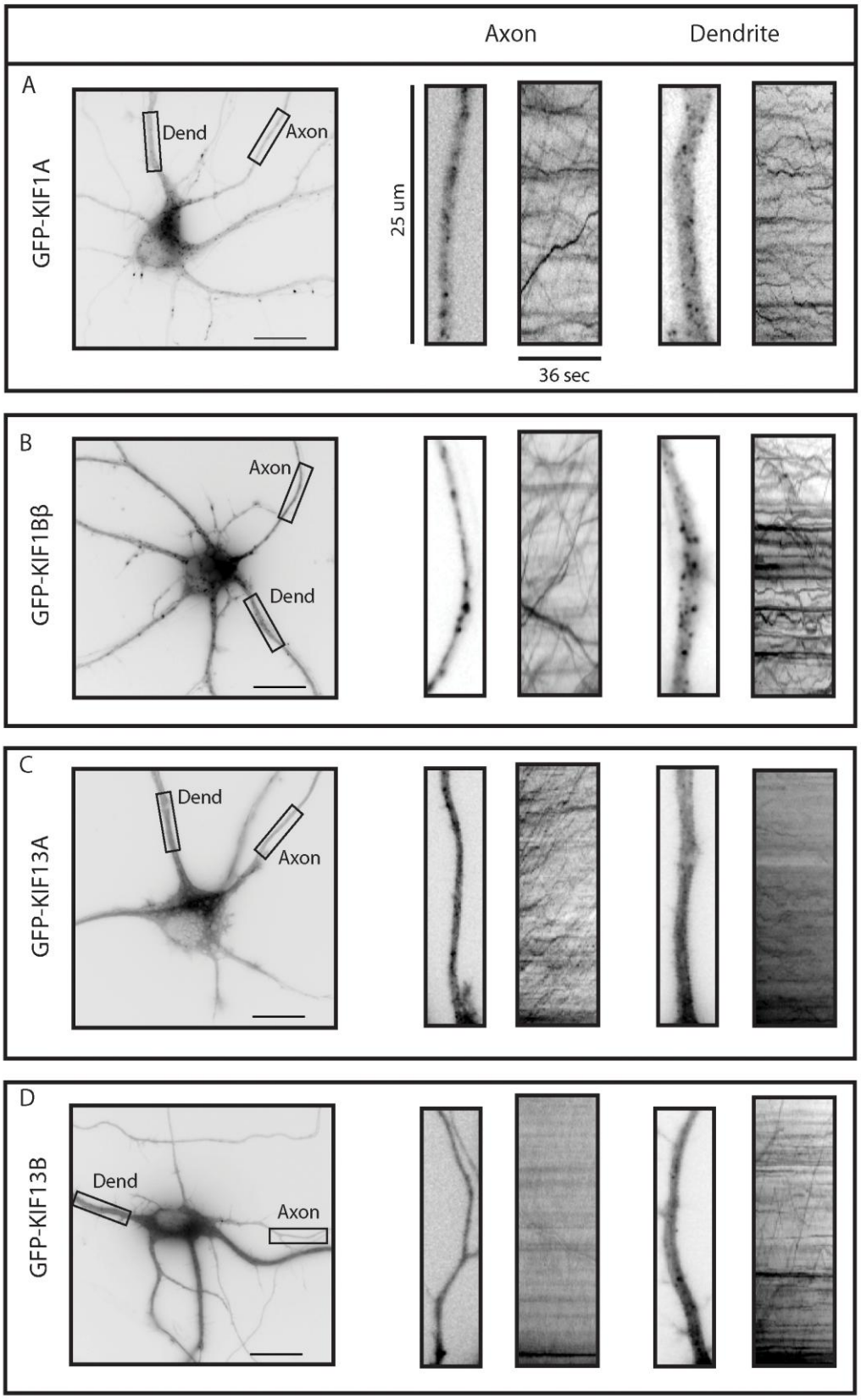

Axon
Dendrite
A
GFP-KIF1A
Dend
Axon
25 um
36 sec
B
GFP-KIF1Bβ
Axon
Dend
C
GFP-KIF13A
Dend
Axon
D
GFP-KIF13B
Dend
Axon

Figure 1: GFP-tagged full-length Kinesin-3 family members expressed in mature hippocampal neurons attached to moving vesicles. Panels **A**-**D** show, from left to right, a low magnification image of a neuron expressing the indicated GFP-kinesin, a high magnification image of an axon and dendrite (boxed in low magnification image) and the corresponding kymograph. Diagonal lines in kymographs with a positive or negative slope indicate transport away and toward the cell body, respectively. Horizontal lines in kymographs indicate GFP-kinesin labeled vesicles that did not move during movie acquisition. Only full-length GFP-KIF13B attaches to vesicles moving exclusively in dendrites, **D**. Contrast of kymographs and images have been inverted so fluorescent signal appears dark on a light background. Scale bar is 20 μm. See chapter 2 supplemental movies 1-4.

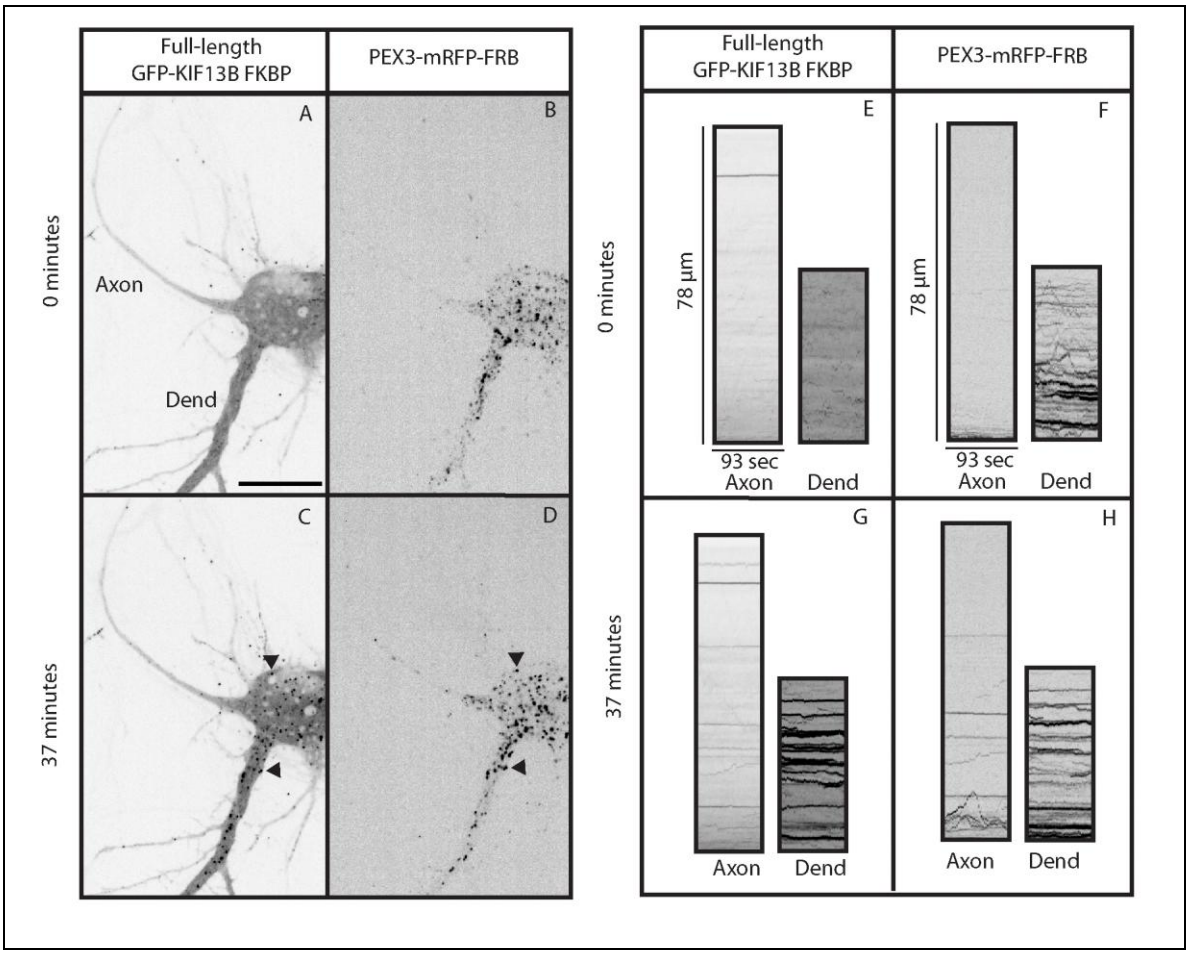

Full-length
GFP-KIF13B FKBP
PEX3-mRFP-FRB
A
B
C
D
0 minutes
37 minutes
Axon
Dend
E
F
G
H
78 µm
93 sec
Axon
Dend

Figure 2: Full-length GFP-KIF13B attaches to but does not transport peroxisomes in mature neurons. Above the panels is an illustration of full-length KIF13B (1-1826aa) with known domains. **A**, GFP-KIF13B is diffuse and not seen attached to vesicles in mature neurons before adding the linker drug. **B**, peroxisomes are distributed throughout the cell body and dendrites of a mature neuron and are relatively motionless before addition of the linker drug. **C**, After addition of the linker drug, GFP-labeled full-length KIF13B has accumulated on the peroxisomes as there are now dark puncta in the cell body that overlap with peroxisomes (Arrowheads). **D**, peroxisomes remain localized to the cell body and dendrites and are not moving – see kymograph of peroxisomes in **H**. **E**-**H**, Kymographs showing peroxisome transport from the axon and one dendrite of cells in **A**-**D**. Horizontal lines in kymographs indicate peroxisomes that did not move during movie acquisition. Scale bar in A is 20 µm. Images **A**-**D** are single frames from a 2-color movie and contrast has been inverted so bright objects appear dark on a light background.

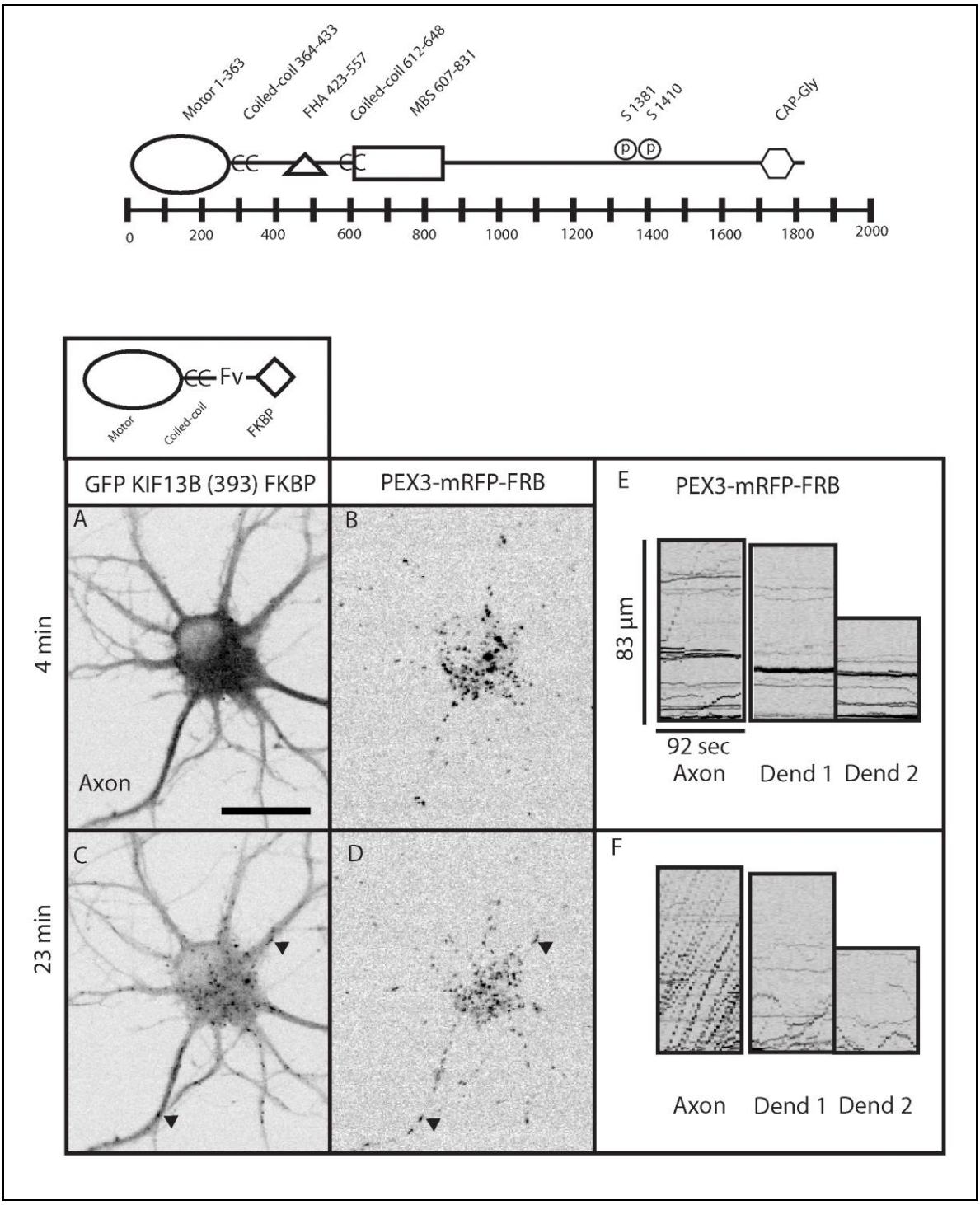

Motor 1-363
Coiled-coil 364-433
FHA 423-557
Coiled-coil 612-648
MBS 607-831
S 1381
S 1410
CAP-Gly
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Fv
Motor
Coiled-coil
FKBP
GFP KIF13B (393) FKBP
PEX3-mRFP-FRB
E
PEX3-mRFP-FRB
A
B
4 min
Axon
83 µm
92 sec
Axon
Dend 1
Dend 2
C
D
F
23 min
Axon
Dend 1
Dend 2

Figure 3: GFP-KIF13B (1-393)-Fv-FKBP attaches to and transports peroxisomes into the axon of mature neurons. Above, a cartoon of full-length KIF13B (1-1826aa) with known domains for comparison to the construct used in the experiment. Drug to link kinesins to peroxisomes was added 15-20 minutes prior to addition of the kinesin homo-dimerization drug. The kinesin homo-dimerization drug was added once the sample was on the microscope. **A**, At 4 minutes post addition of the kinesin homo-dimerization drug, GFP-KIF13B motor domain (1-393aa) FKBP was diffusely distributed showing the cell's morphology. **B**, At 4 minutes post addition of the kinesin homodimerizaiton drug PEX3-mRFP-FRB-labeled peroxisomes are mostly motionless and found in the cell body and proximal axon and dendrites. **C**, 23 minutes after addition of the kinesin homo-dimerization drug green puncta are apparent indicating the motor domain has accumulated onto the peroxisomes (arrowheads indicate overlap with peroxisomes in **D**). **D**, Image of peroxisomes at 23 minutes. **E** and **F** show kymographs from the axon and two dendrites from the cell pictured at indicated timepoints. **E**, Peroxisomes show little transport 4 minutes after drug addition. **F**, Kymographs of the axon and two dendrites show numerous peroxisomes transported into the axon with few long distance transport events in the dendrites. Scale bar is 20µm. Images **A**-**D** are single frames from a 2-color movie and contrast has been inverted. See chapter 2 supplemental movie 5.

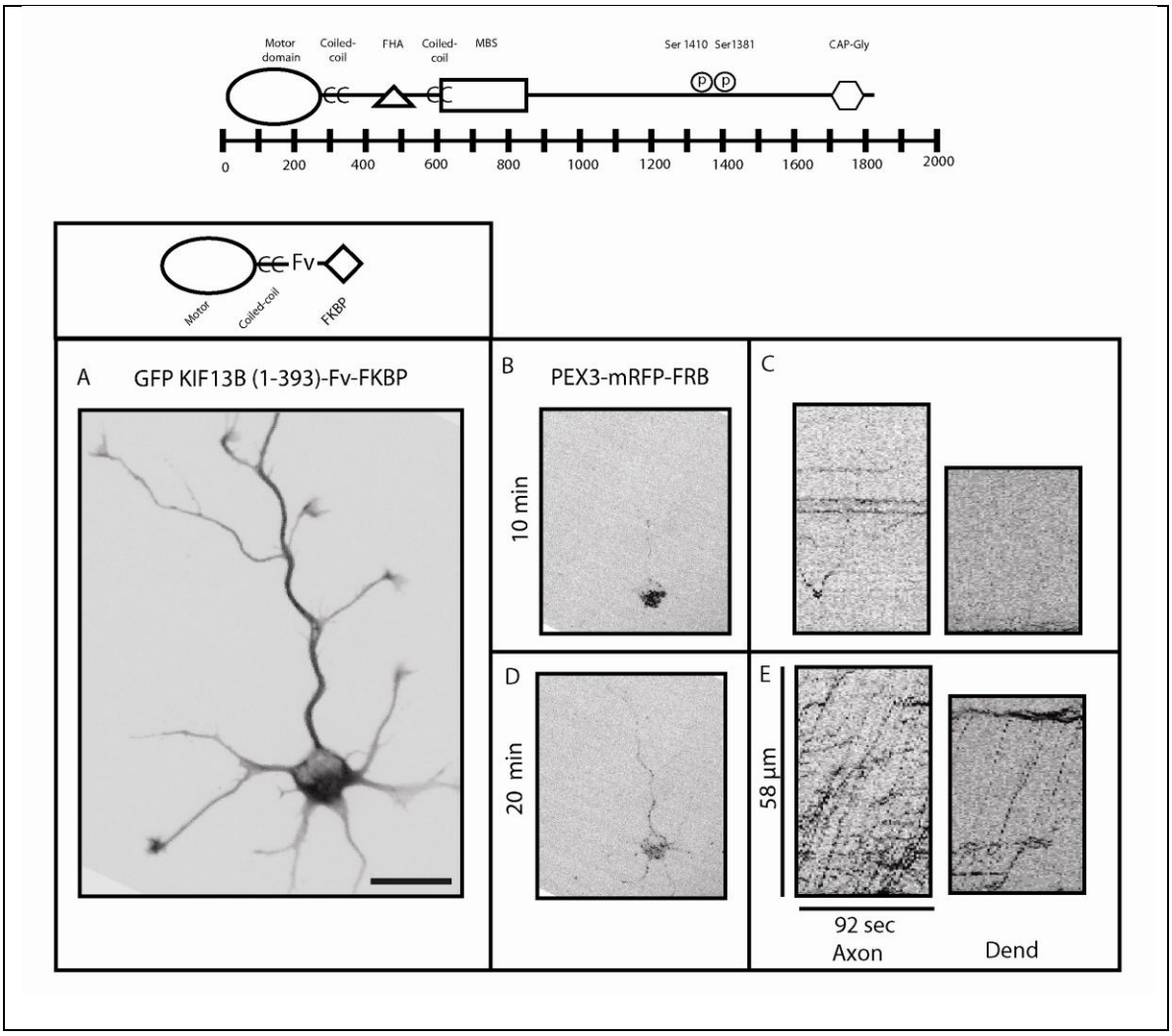

Motor domain
Coiled-coil
FHA
Coiled-coil
MBS
Ser 1410 Ser1381
CAP-Gly
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Fv
Motor
Coiled-coil
FKBP
A
GFP KIF13B (1-393)-Fv-FKBP
B
PEX3-mRFP-FRB
10 min
C
D
20 min
E
58 µm
92 sec
Axon
Dend

Figure 4: GFP-KIF13B (1-393) motor domain attaches to and transports peroxisomes into the axon and dendrites of young stage 3 neurons. **A**, Diffuse distribution of GFP-KIF13B motor domain before peroxisome transport shows the neuron's morphology. **B**, **D**; Images show peroxisome localization before and after peroxisome transport begins. **B**, Demonstrates the peroxisomes are localized to the cell body before peroxisome transport. **D**, Peroxisomes are redistributing into the axon and dendrites after 20 minute exposure to the linker drug. **D**, **E**, Kymographs of peroxisome transport created from 2-color, time-lapse movies of the axon and two dendrites from the cell pictured at the indicated time points. Only the peroxisome signal was used for the kymographs. **E**, Positively sloped lines in the axon and dendrites indicate peroxisome transport. Horizontal lines at the top of dendrite kymographs indicate peroxisomes have accumulated at the tips of dendrites. The peroxisomes at the tips do not return to the cell body. Scale bar is 20µm. Images **A**, **B** and **D** are from 2-color movies and contrast has been inverted. See chapter 2 supplemental movie 6.

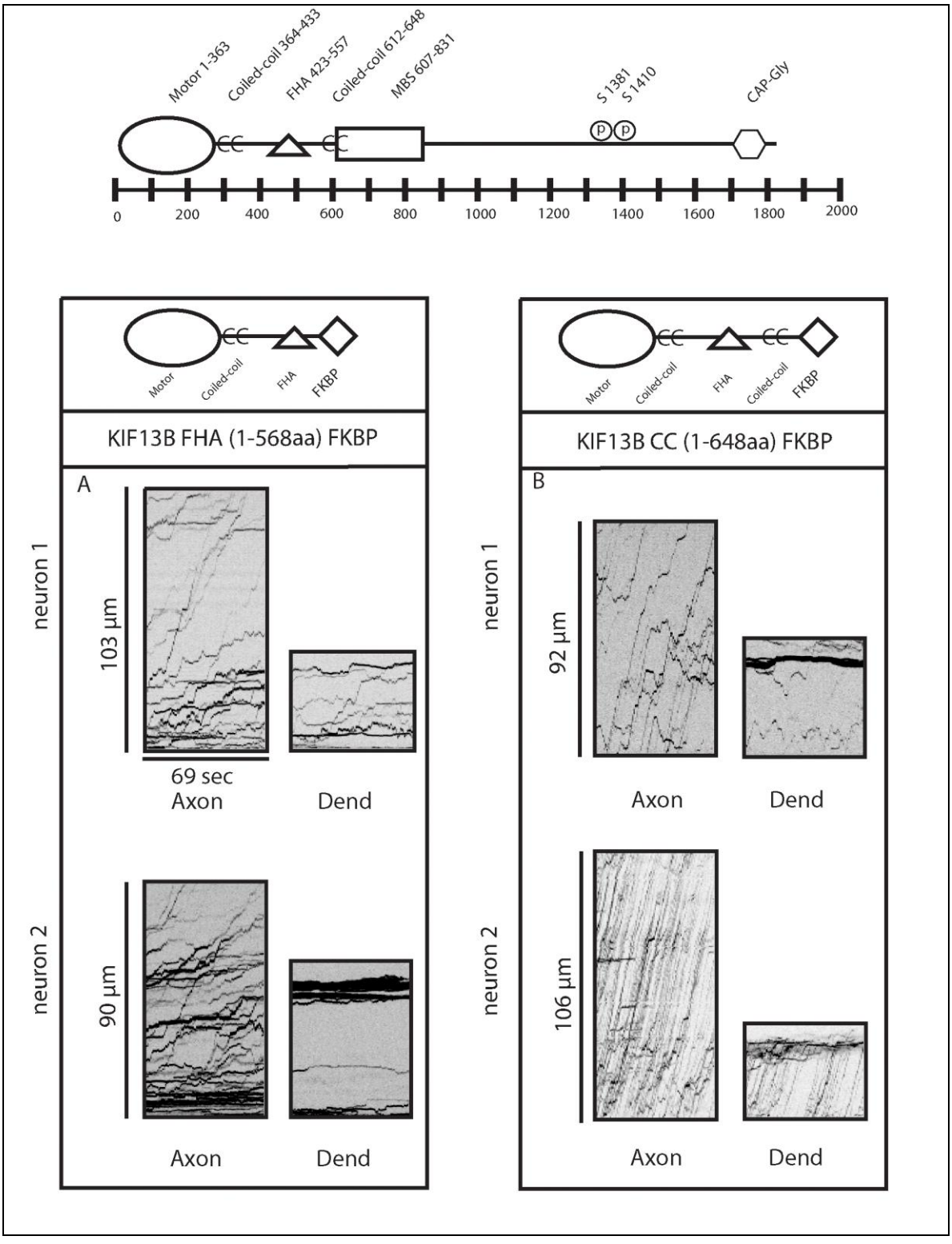

Motor 1-363
Coiled-coil 364-433
FHA 423-557
Coiled-coil 612-648
MBS 607-831
S 1381
S 1410
CAP-Gly
P
P
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Motor
Coiled-coil
FHA
FKBP
KIF13B FHA (1-568aa) FKBP
A
neuron 1
103 µm
69 sec
Axon
Dend
neuron 2
90 µm
Axon
Dend
Motor
Coiled-coil
FHA
Coiled-coil
FKBP
KIF13B CC (1-648aa) FKBP
B
neuron 1
92 µm
Axon
Dend
neuron 2
106 µm
Axon
Dend

Figure 5: KIF13B truncation and deletion constructs transport peroxisomes non-selectively but with different rates and processivities. **A**-**D** show representative kymographs of the axon and one dendrite of young stage 3 neurons co-expressing the indicated KIF13B constructs and the PEX3 protein. Two neurons from each construct are shown to display cell to cell variation. **A**, Most cells expressing GFP-KIF13B FHA (1-568aa) transported peroxisomes with varying levels of processivity as shown by the kymographs. **B**, most cells expressing GFP-KIF13B CC (1-648aa) transported peroxisomes more processively than KIF13B 1-569aa. See chapter 2 supplemental movies 7 and 8.

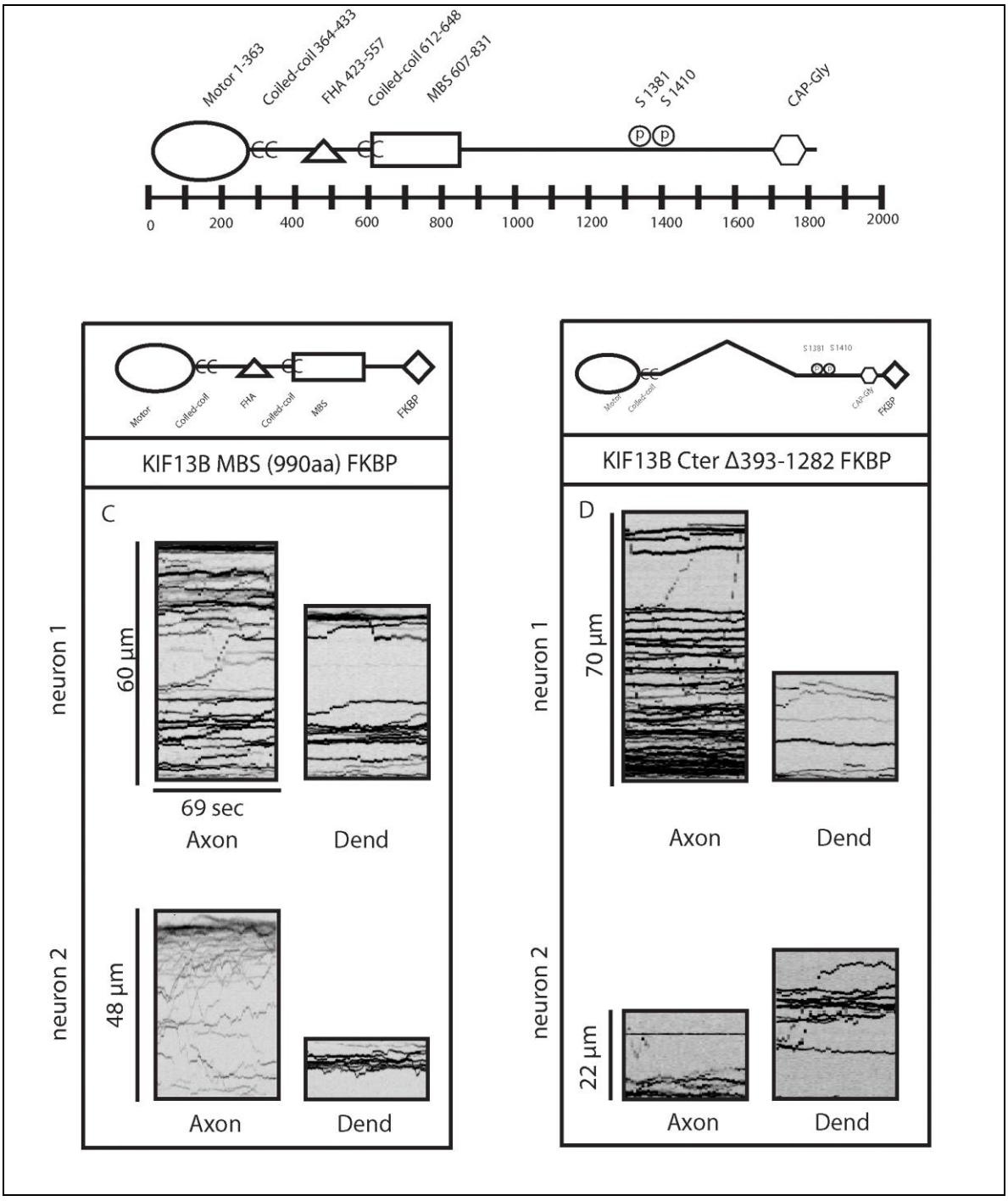

Motor 1-363
Coiled-coil 364-433
FHA 423-557
Coiled-coil 612-648
MBS 607-831
S 1381
S 1410
CAP-Gly
P
P
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Motor
Coiled-coil
FHA
Coiled-coil
MBS
FKBP
KIF13B MBS (990aa) FKBP
C
neuron 1
60 µm
69 sec
Axon
Dend
neuron 2
48 µm
Axon
Dend
S1381 S1410
Motor
Coiled-coil
CAP-Gly
FKBP
KIF13B Cter Δ393-1282 FKBP
D
neuron 1
70 µm
Axon
Dend
neuron 2
22 µm
Axon
Dend

Figure 5 cont. **C**, Most cells expressing GFP-KIF13B MBS (1-990aa) carried peroxisomes to the tips of axons and dendrites but few long-distance, processive transport events were captured live (16cells). **D**, most cells expressing GFP-KIF13B C-terminus (Δ393-1282aa) transported peroxisomes to the tips of axons and dendrites but few long-distance, processive transport events were captured live. See chapter 2 supplemental movie 9.

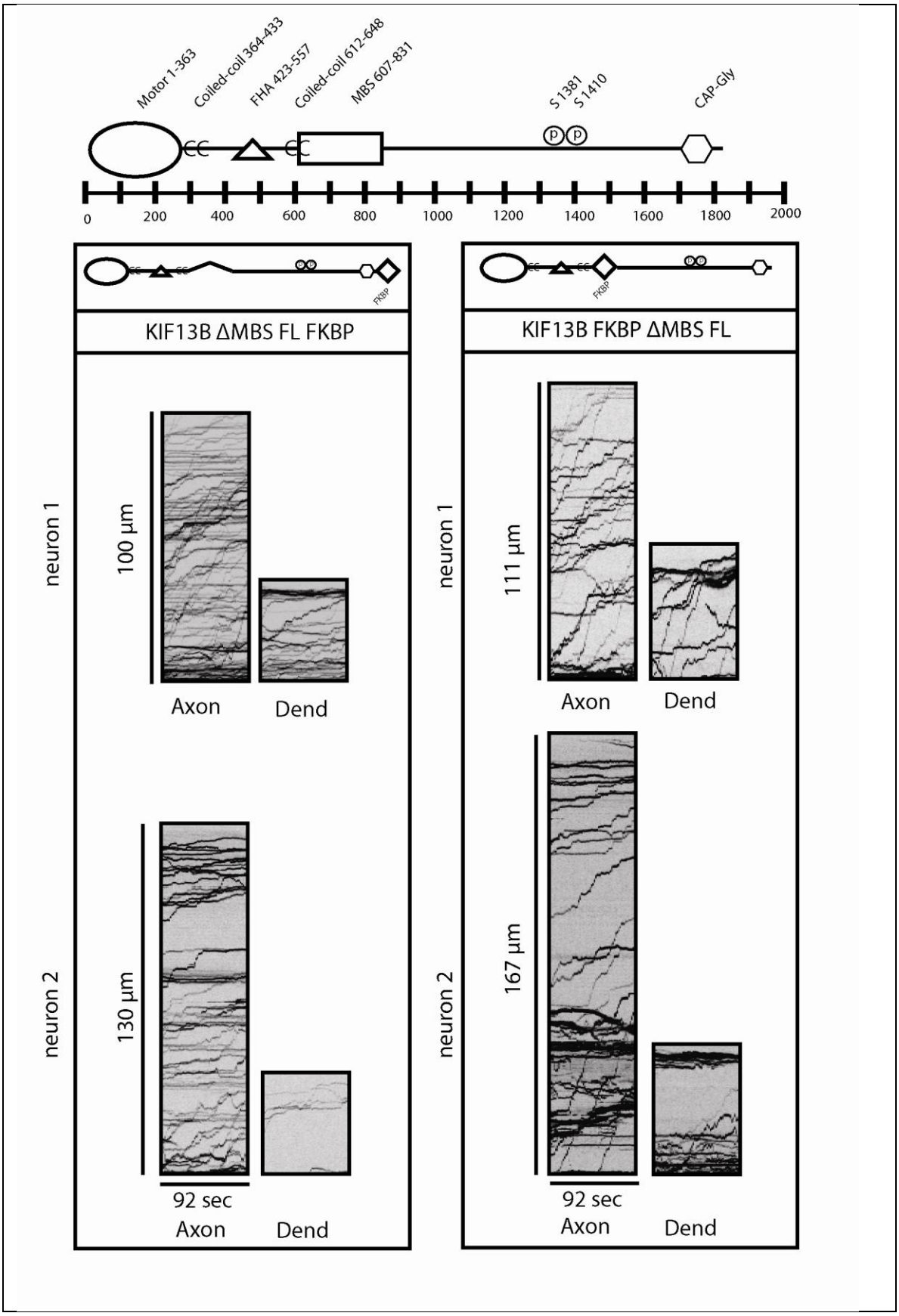

Motor 1-363
Coiled-coil 364-433
FHA 423-557
Coiled-coil 612-648
MBS 607-831
S 1381
S 1410
CAP-Gly
P
P
0
200
400
600
800
1000
1200
1400
1600
1800
2000
FKBP
KIF13B ΔMBS FL FKBP
neuron 1
100 µm
Axon
Dend
neuron 2
130 µm
92 sec
Axon
Dend
FKBP
KIF13B FKBP ΔMBS FL
neuron 1
111 µm
Axon
Dend
neuron 2
167 µm
92 sec
Axon
Dend

Figure 6: KIF13B constructs lacking the MBS transported peroxisomes into axons and dendrites of young stage 3 neurons. **A**, Kymographs of neurons expressing GFP-KIF13B ΔMBS FKBP show this construct transported peroxisomes in axon and dendrites. Most peroxisomes moved in a slow, non-processive fashion while some exhibited long distance processive events. **B**, Kymographs of neurons expressing GFP-KIF13B FKBP ΔMBS show this construct transported peroxisomes in axons and dendrites. Most peroxisomes moved in a slow non-processive manner but more examples of long-distance, processive events could be found when compared to the deletion construct in **A**. See chapter 2 supplemental movie 10 and 11.

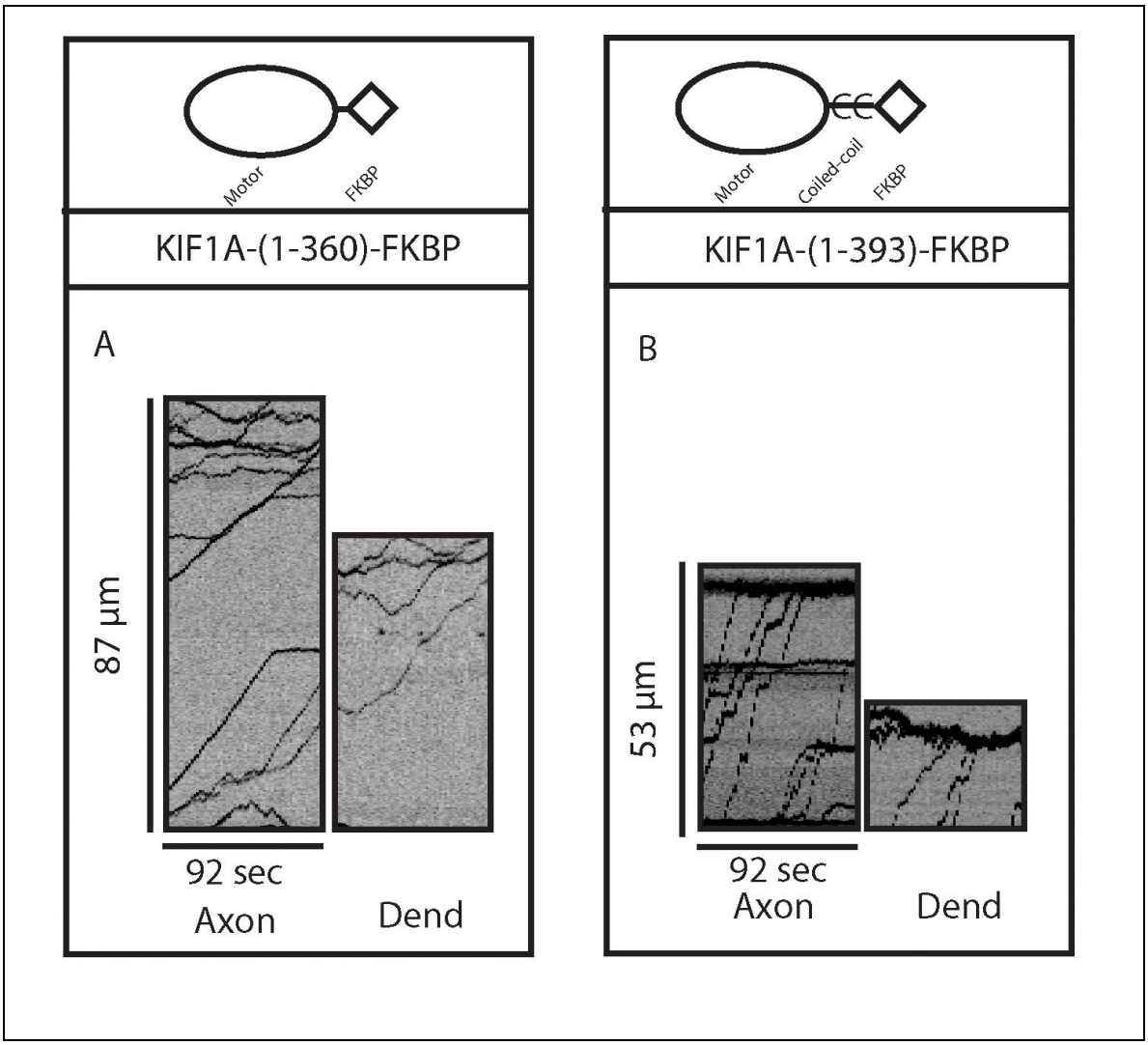
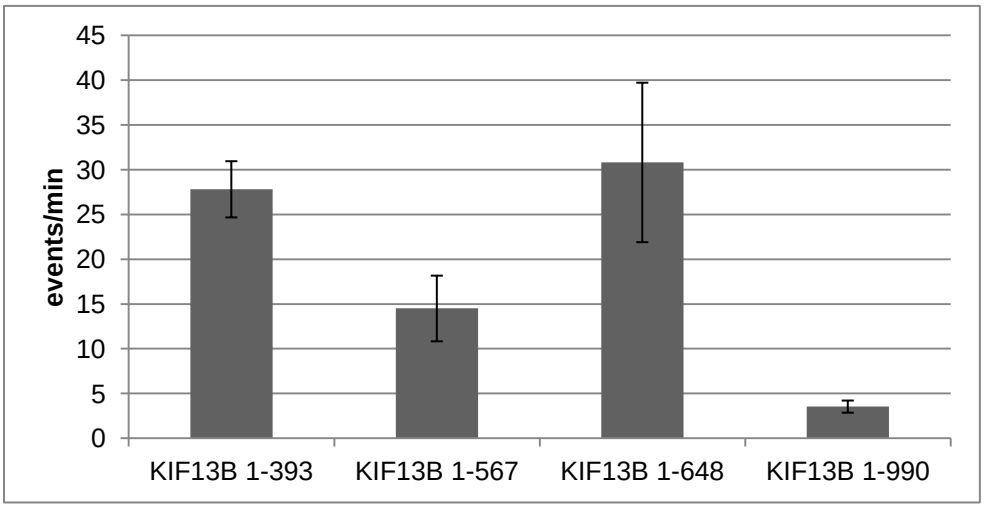

Motor
FKBP
KIF1A-(1-360)-FKBP
A
87 µm
92 sec
Axon
Dend
Motor
Coiled-coil
FKBP
KIF1A-(1-393)-FKBP
B
53 µm
92 sec
Axon
Dend

Figure 7. KIF1A transports peroxisomes as a monomer and a dimer. **A**, Kymographs of peroxisome transport in the axon and one dendrite of a neuron expressing a monomeric version of KIF1A (1-360). The monomer transports peroxisomes on average at 0.6 µm/sec. **B**, Kymographs of peroxisome transport in the axon and one dendrite of a neuron expressing a dimeric version of KIF1A. The KIF1A dimer transports peroxisomes at 2.1 µm/sec. See chapter 2 supplemental movies 12 and 13.

A



B

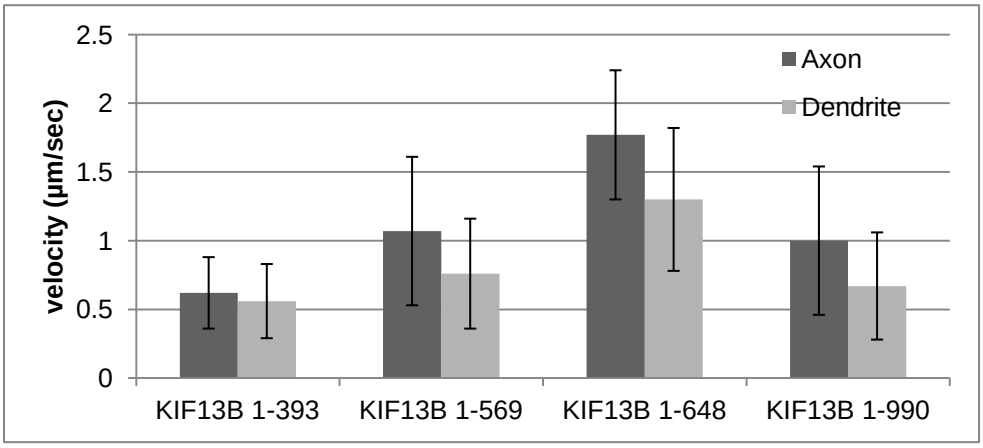


Figure 8. KIF13B-mediated peroxisome transport. A, Graph showing mean number of peroxisome events that traveled greater than 3µm in the axon. B, mean velocity of peroxisome transport in the axon and dendrites of stage 3 neurons expressing the indicated construct.

A

construct	# cells	mean velocity (um/sec) +/- SD	mean run length (um) +/- SD	mean events >3um / axon
KIF13B 1-393	10	0.62 +/- 0.26	9.8 +/- 6.6	27.80
KIF13B 1-569	6	1.07 +/- 0.54	5.0 +/- 4.4	14.50
KIF13B 1-648	5	1.77 +/- 0.47	12.9 +/- 8.6	30.80
KIF13B 1-990	10	1.0 +/- 0.54	5.0 +/- 5.1	3.54
KIF13B d393-1293	nd	nd	nd	nd
KIF13B dMBS FL FK	5	0.65 +/- 0.33	5.7 +/- 3.8	nd
KIF13B FK dMBS FL	3	0.8 +/- 0.43	5.9 +/- 4.4	nd
KIF13B FL	4	N/A	N/A	N/A
KIF1A 1-360	3	0.61 +/- 0.33	7.24 +/- 6.4	nd
KIF1A 1-393	3	2.1 +/- 1.1	9.9 +/- 6.8	nd

B

construct	#cells/neurites with events	mean velocity (um/sec) +/- SD	mean run length (um) +/- SD	mean events >3um/dendrite
KIF13B 1-393	5/15	0.56 +/- 0.27	6.6 +/- 4.2	8.9
KIF13B 1-569	3/7	0.76 +/- 0.4	3.2 +/- 1.7	9.1
KIF13B 1-648	4/9	1.3 +/- 0.52	5.6 +/- 3.14	15.8
KIF13B 1-990	2/6	0.67 +/- 0.39	1.9 +/- 0.96	0.5
KIF13B d393-1293	nd	nd	nd	nd
KIF13B dMBS FL FK	1/3	0.57 +/- 0.33	4.2 +/- 2.7	nd
KIF13B FK dMBS FL	1/4	0.8 +/- 0.45	6.2 +/- 3.8	nd
KIF13B FL	N/A	N/A	N/A	N/A
KIF1A 1-360	2/5	0.8 +/- 0.36	5.4 +/- 3.5	7.4
KIF1A 1-393	1/2	2.45 +/- 1.1	7.6 +/- 3.9	17

Table 1. Table of peroxisome transport mediated by various constructs of KIF13B and two KIF1A constructs. A, Axonal transport. B, Dendritic transport. (N/A = not applicable; nd = not determined)

Materials and methods

Live cell imaging

During imaging, cells were maintained at 32–34°C in a heated chamber (Warner Instruments, Hamden, CT) containing Hibernate E without phenol red (BrainBits, Springfield, IL) supplemented with B27 (Invitrogen, Carlsbad, CA). Objectives were warmed to 34°C (Bioptechs Inc.). Live images were acquired on a Nikon TE2000 with a spinning-disk, confocal-head (Solamere Technology Group; Yokogawa CSU10) with an Orca-ER CCD camera (Hamamatsu Photonics Co., Ltd). A Nikon Plan-Apo 60x 1.45 NA objective was used to acquire movies. During movie acquisition Z-axis movement was controlled by Nikon TE2000 Perfect Focus. Movies were acquired by streaming 60-90 frames (600-750ms/frame) before drug treatment and at 10-15min intervals after drug treatment.

Movies for figure 1 (Chapter 2 and Chapter 3) were acquired on a Leica DM RXA (Leica Microsystems, Wetzlar, Germany) with a Micromax CCD camera (Princeton Instruments) using a 63x Plan-Apo 1.32 objective. Cells were maintained as above but using imaging media consisting of HBSS with Ca^{2+}, Mg^{2+}, and 10mM HEPES (Gibco) with 0.6% glucose. MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to drive microscopes and analyze movies.

Cell Culture

Primary hippocampal cultures were prepared from embryonic day 18 rats and maintained in MEM with N2 supplements, and transfected as described previously [94]. Constructs were transfected into Stage 4 hippocampal neurons (8-12 days in culture) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and expressed for 8-18 h before imaging.

Constructs

Most KIF13B and all KIF1A constructs all had a GFP fused in frame on the N-terminus and the FKBP moiety fused in frame to their C-terminus. GFP-KIF13B 1-648 dMBS FKBP -1826 had the FKBP moiety fused in frame at aa648. See table.

For mature neurons (figures 1 and 2) cultured hippocampal neurons were transfected at DIV 8-10 using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were allowed to express the construct for 18-24 hours before imaging. Stage 3 neurons (figures 3-7) cultured hippocampal neurons were electroporated with the PEX3-tdTomato-FRB and a kinesin construct using the Amaxa electroporation system (Lonza) according to the manufacturer's protocol.

The linkage of the KIF13B construct to the peroxisome construct was induced by treating cells expressing both constructs with AP 21967, a rapamycin analog as used in Kapitein et al [57]. Ariad Pharmaceuticals (Clontech). Homo-dimerization of the kinesin motor domains were used as previously described in [23] and according to the manufacturer's protocol (Ariad).

Constructs used
KIF13B constructs
GFP-1-393-FKBP
GFP-1-569-FKBP
GFP-1-648-FKBP
GFP-1-990-FKBP
GFP-1-393-(Δ394-1292)-1826-FKBP
GFP-1-648 (FKBP) 990-1826
GFP-1-648 –(Δ649-990)-1826-FKBP
GFP-1-1826-FKBP
KIF1A constructs
GFP-1-360-FKBP
GFP-1-393-FKBP
Peroxisome construct
PEX3-tdTomato-FRB

Chapter 3: A novel method to identify the kinesins that mediate dendritic vesicle transport

Introduction

Long-range intracellular transport towards the plus-end of microtubules is principally carried out by kinesin family motor proteins. All kinesin family members are structured similarly, with a globular “motor domain” that uses ATP hydrolysis to translocate along microtubules and a “tail domain” that interacts with cargo vesicles [95]. While the mechanics of motor domain translocation are relatively well understood, much less is known about how kinesins interact with their vesicular cargoes and how these kinesin-vesicle interactions direct transport to the correct intracellular destinations.

In the case of nerve cells, directed long-range transport is thought to be crucial for the maintenance of neuronal polarity, ensuring that axons and dendrites acquire their correct complement of membrane proteins. Live-cell imaging of cultured neurons shows that vesicle transport is selective. Vesicles containing dendritic proteins are transported efficiently into the dendrites but do not enter the axon [6, 96]. Vesicles containing axonal membrane proteins are not excluded from dendrites, but their transport is biased toward the axon [6, 97].

Several different models have been proposed to account for dendrite-selective transport. Some models propose that dendritic vesicles are transported by “smart” kinesins that can distinguish dendritic from axonal microtubules [6, 98, 99]. Other models suggest that the kinesin-driven transport of dendritic vesicles is arrested when they enter the axon initial segment [47, 58]. Yet another model argues that dendritic transport is mediated exclusively by dyneins, without the involvement of any kinesins [57]. Biochemical strategies have identified

associations between kinesins, scaffolding proteins, and dendritic membrane proteins, but it has been difficult to establish the role of specific kinesins by direct assays of dendritic vesicle transport [18, 48, 95]. Nor has there been an unbiased screen to determine whether different kinesins contribute to the transport of a single vesicle population. Until we know which kinesins mediate dendrite-selective transport, or whether kinesins are even involved, it will be difficult to determine the mechanisms that confer selectivity.

Here we introduce a novel, live cell-imaging assay capable of identifying the kinesins that interact with a fluorescently labeled vesicle population and use this approach to determine the kinesins that participate in dendrite-selective transport. This “split-kinesin” method entails expressing separate constructs encoding a kinesin tail and a kinesin motor domain that can be assembled into a fully functional kinesin using a linker drug. The kinesin tail binds vesicles, but is incapable of influencing their movement because it lacks a motor domain; the motor domain walks constitutively along microtubules, but cannot move vesicles because it lacks a cargo binding domain. When the two components are linked together, their assembly leads to a rapid and profound change in the trafficking pattern of only the vesicles that bind the expressed kinesin tail domain.

Using this strategy we identify three kinesins that mediate the selective transport of dendritically polarized cargoes, including two little-known members of the Kinesin-3 family, KIF13A and KIF13B, whose role in dendritic transport has not been previously suspected. Intriguingly, two of the three dendritic kinesins also mediate the *axonal* transport of other vesicle populations, confirming the

hypothesis that kinesins can be steered to the axon or the dendrites depending on whether they interact with an axonal or a dendritic cargo [36]. We also show that when dendritic vesicles are mis-directed to the axon, there is no fail-safe mechanism that prevents their fusion with the axonal plasma membrane. This result confirms that selective transport is a primary mechanism for maintaining neuronal polarization.

Results

Vesicle populations labeled by GFP-tagged Kinesin-3 family members

Neurons express some 15 different kinesins thought to mediate vesicle or organelle transport [100]. In a screen to identify candidate kinesins that mediate axon- or dendrite- selective transport, Huang and Banker expressed constitutively active motor domains of all these kinesins in cultured neurons [101]. This work demonstrated that most kinesin motor domains accumulate only in axonal tips [54, 97, 102]. However, the Kinesin-3 family members KIF1A, KIF1Bβ, and KIF13B, accumulate in both axon and dendrite tips, as does the Kinesin-4 family member KIF21B [101]. These results indicate that members of the Kinesin-3 and Kinesin-4 families merit further investigation as potential motors for dendritically polarized vesicles. To further explore this possibility, we expressed full-length, N-terminally GFP-tagged versions of these kinesins in cultured hippocampal neurons in order to observe the localization and movements of vesicles they labeled. While most kinesin motors do not label vesicles, some members of the Kinesin-3 family label vesicles well enough for live cell imaging [103].

Figure 1 shows representative examples of cells expressing GFP-tagged members of the Kinesin-3 family (In this and all subsequent figures, the contrast has been reversed so that fluorescent objects appear dark on a light background). The corresponding kymographs show the movement of the labeled vesicles in these cells and compare their patterns of transport. In the kymographs, moving vesicles give rise to diagonal lines; lines with a positive

slope represent movements away from the cell body. The most consistent vesicle labeling was seen following expression of KIF1A and Kif1Bβ, but in favorable cases it was possible to image vesicles labeled by KIF13A and KIF13B. KIF1A, KIF1Bβ, and KIF13A all label vesicles that are transported bi-directionally in axon and dendrites (Figure 1A-C). In contrast, vesicles labeled with KIF13B were largely polarized to the dendrites (Figure 1D). During live recordings, KIF13B-labeled vesicles rarely entered the axon, but were transported efficiently in the somatodendritic domain of the cell. GFP-tagged Kinesin-4 family members showed a diffuse distribution when expressed in cultured neurons, making it impossible to observe vesicle behavior (data not shown).

These results indicate that KIF13B could be a purely dendritic vesicle motor. The remaining Kinesin-3 motors likely mediate transport of both dendritic and axonal cargo, but are not inherently targeted to a particular domain of the cell.

Attaching a constitutively active axonal kinesin motor domain to a labeled vesicle population results in a substantial increase in axonal transport

To further investigate the role of these and other kinesins in dendrite-selective transport, we sought an unbiased strategy to identify all of the kinesins that contribute to the transport of a given population of vesicles. The idea behind our approach involves the construction of a library of chimeric “split kinesins,” created by combining a kinesin motor domain that displays a strong bias for transport to the axon with the cargo binding tails from all of the candidate kinesins that might mediate the transport of dendritic vesicles. We engineered a

constitutively active construct of the KIF5C motor domain ($KIF5C^{560}$) fused to the FK506 binding protein (FKBP) and a series of kinesin tails fused to the FKBP12-rapamycin binding (FRB) domain [54, 88]. The FKBP and FRB domains can be inducibly linked in the presence of the rapamycin analog AP21967. Kapitein and colleagues used this approach to directly link a constitutively active kinesin motor domain to a peroxisomal membrane protein, which led to a pronounced increase in peroxisome movement within minutes [57]. If the split kinesin strategy were successfully applied to a dendritic vesicle population, addition of the linker drug would result in the rapid assembly of active, axon-selective kinesins, producing a wave of vesicles traveling into the previously unlabeled axon.

As a proof of principle, we attempted to alter the behavior of the vesicles that bind KIF1A. We expressed a GFP-KIF1A tail domain fused to the FRB domain together with the KIF5C motor domain fused to FKBP. Figure 2A shows the pattern of transport of vesicles labeled with fluorescently tagged KIF1A tail before and after the addition of the linker drug (Supplemental movie 1). Kymographs before addition of the linker drug showed vesicles moving bi-directionally in both the axon and the dendrite. These transport events were mostly short and there were relatively few of them. Labeled vesicles move as efficiently in axon and dendrite before the addition of the linker drug. After adding the rapamycin analog, the axonal transport of KIF1A-labeled vesicles increased markedly (Figure 2A). A continuous stream of vesicles entered the axon and moved for long distances without pauses or reversals. The increase in transport began about 15 minutes after treatment and peaked between 20 and 30 minutes.

In the dendrites there was no change in transport. This confirms that attaching the constitutively active KIF5C^{560} motor domain to vesicles that bind the KIF1A tail domain drastically changes their trafficking behavior.

To confirm that this strategy can increase the axonal transport of a well-characterized vesicle population visualized by expressing a labeled cargo protein, we investigated the trafficking of secretory granules labeled by expressing Brain-derived Neurotrophic Factor (BDNF) [104]. Previous work has established that knockdown of KIF1A results in a reduction in BDNF transport, implying that this kinesin participates in the transport of secretory granules [105]. For this experiment BDNF-RFP, FRB-KIF1A tail, and KIF5C^{560}-FKBP were expressed together (Figure 2B). Consistent with previous results, kymographs recorded before addition of linker drug show that BDNF-positive vesicles are transported bi-directionally in both axons (Figure 2B) and dendrites (data not shown); [105]. Their movements were mostly short. After the addition of linker drug, there was a clear increase in transport in the axon. Many more secretory granules entered the axon, where they traveled at a constant velocity and could be followed for many tens of micrometers.

In some cases the over-expression of kinesin tails results in dominant negative effects [106]. In our experiments, expressing the KIF1A tail did not cause a reduction in the movements of BDNF granules (data not shown) and did not result in fewer vesicle movements than were seen after expressing full-length KIF1A.

These experiments indicate that it is possible to drastically change the trafficking behavior of cargo vesicles by allowing the appropriate kinesin tail domain to interact with a specific vesicle population, then chemically linker this tail to a constitutively active motor domain.

KIF13A and KIF13B bind Transferrin Receptor vesicles

Having established the feasibility of this approach, we attempted to use this strategy to identify the kinesin tails that interact with vesicles that undergo dendrite-selective transport. Compared with axonal vesicles, dendrite-selective vesicles are small and dim and the presence of labeled proteins undergoing synthesis in the dendritic rough endoplasmic reticulum increases background labeling. We chose to label dendritic vesicles by expressing GFP-tagged Transferrin Receptor (TfR), which provides a comparatively high signal to noise ratio, making it possible to image extensive transport in dendrites [6, 96]. TfR is highly polarized to dendrites in both vertebrate and invertebrate neurons, which is reflected in the movement of TfR containing vesicles; more than ten times as many vesicle movements are observed in a typical dendrite compared with the axon [6, 52, 96, 98]. Vesicles labeled with GFP-TfR are thought to contain other dendritically polarized proteins as well, including AMPA receptors [107].

To identify kinesins that bind TfR vesicles, we co-expressed KIF5C^{560}-FKBP and a series of kinesin tails that had an N-terminal FRB domain and a myc tag (Figure 3A). Staining for myc confirmed that more than 90 % of cells that were labeled with TfR-GFP co-expressed all three constructs (data not shown).

Cells were then incubated with linker drug while movies of the TfR-GFP were collected.

Figures 3B-D show a cell expressing TfR-GFP, KIF5C^{560}, and the tail of KIF13B, a member of the Kinesin-3 family. Before adding the drug, there was robust dendritic transport of TfR, but little transport in the axon. By 9 min after drug addition, the transport pattern showed the characteristic increase in vesicles undergoing high-velocity, long-range anterograde movement. The trafficking increase reached its maximum between 16 and 23 min (Supplemental movie 2). Throughout this period, there was no increase in the number of retrograde events in the axon. Interestingly, the overall intensity of the proximal axon also increased during this period, as can be seen in the kymographs and the still image (Figure 3D). This result could indicate that some of the TfR vesicles that entered the axon fused with the plasma membrane.

Figures 3E-L show kymographs representing axonal transport of TfR-GFP in cells expressing a series of different split kinesins. The screen included all five members of the Kinesin-3 family that are expressed at high levels in neurons, including both of the prominent splice variants of KIF1B (Extended Experimental Procedures Table 1). We also evaluated three other kinesins that have been implicated in dendritic transport: the conventional Kinesin-1 family member KIF5C, which is thought to interact with AMPA receptors [36]; the Kinesin-2 family member KIF17, which has been implicated in the dendritic transport of NMDA receptor subunits and the $Kv_{4.2}$ potassium channel [19, 48]; and KIF21B, the Kinesin-4 family member that accumulates at both dendritic and axonal tips in

the truncated kinesin expression assay [101]. Prior to adding the linker drug there was robust dendritic TfR transport in all cells, which demonstrates that expression of these kinesin tails did not result in dominant negative effects. After adding the linker, both KIF13A and KIF13B tails gave a strong positive interaction with TfR vesicles, as indicated by the marked enhancement in anterograde axonal transport. Expression of KIF1A, KIF1Bα, KIF1Bβ, KIF5C, KIF17, and KIF21B tails all failed to produce any detectable increase in the movement of TfR vesicles in the axon. This demonstrates the high specificity of this experimental paradigm.

In all, each tail construct was screened in seven to 20 cells (Table 1). Results were scored as positive if addition of the linker drug resulted in the characteristic surge in the number of vesicles undergoing long-range anterograde transport in the axon. To reduce the possibility of false-negative results, cells were included in this analysis only if TfR was well polarized (to ensure that an increase in axonal traffic would be obvious) and if the cell displayed robust dendritic transport both before and after addition of the linker drug (to ensure that the transport machinery was fully functional). Only cells expressing KIF13A or KIF13B showed any increase in axonal traffic; in both cases, 55%-65% of the cells assayed showed a strong increase in trafficking. Given the complexity of the assay, which requires expression of multiple constructs and chemical cross-linking between constructs, it is not surprising that some cells expressing KIF13A or KIF13B tails yielded negative results.

To quantify changes in transport of TfR vesicles in cells expressing different tails, we prepared kymographs of vesicle transport before and 20-30 minutes after adding the linker drug. To reduce the possibility of false-negative results, cells were included in this analysis only if TfR was well polarized (to ensure that an increase in axonal traffic would be obvious) and if the cell displayed robust dendritic transport both before and after addition of the linker drug (to ensure that the transport machinery was fully functional). The results are shown in Fig. 6A, which indicates the difference in the number of anterograde events observed in the axon after adding the linker drug for each of the expressed tails There was a prominent, statistically significant increase in axonal transport in cells expressing KIF13A and KIF13B (test and p values). In cells expressing the other kinesin tails there was a small decrease in transport 20-30 minutes after adding the linker drug. This likely reflects a slight inhibition of transport due to photo-toxicity as a result of the extensive imaging required to quantify transport (typically 180 to 240 exposures over 30 minutes).

Altered kinesin-driven transport of TfR vesicles causes accumulation of mis-localized TfR on the axonal surface

Having identified two kinesin tails that interact with TfR vesicles, we wanted to determine if prolonged activation of a KIF13B split kinesin leads to a detectable increase in axonal TfR and whether the mis-targeted protein is able to

reach the axonal surface. It is unknown whether dendritic vesicles can fuse with the plasma membrane in the axon, or if there is a fail-safe mechanism that prevents the fusion of dendritic vesicles with the axonal membrane. Misdirecting dendritic vesicles into the axon and measuring the surface arrival of the cargo protein will provide an answer to this question.

To accomplish this, cells expressing TfR-GFP, KIF5C^{560}-FKBP, and FRB-KIF13B tail were treated with linker drug for 4 h. The total amount of TfR-GFP in the axon was assessed by measuring GFP fluorescence and the TfR-GFP that had reached the cell surface was detected by staining living cells with antibodies against GFP (present in the ectodomain of TfR). Figure 4A shows a control cell (not exposed to the linker drug) and a treated cell. The control cell displayed strong GFP fluorescence and cell surface immunostaining in the dendrites, but not in the axon. A higher magnification view of the boxed region shows how little TfR is present in the axon. In the cell treated with linker drug for 4 h, a strong punctate TfR signal is also present in the axon. The high magnification inset shows an increase in both the diffusely distributed signal intensity of the axon, as well as an accumulation of TfR in axonal aggregates. Many of these bright objects could be labeled from the cell surface, as indicated by the live staining, and probably represent endosomes that formed while the cells were exposed to anti-GFP antibody.

Figure 4B shows line scan quantifications of the boxed region of the axon for the control cell and treated cells. While there is little variation in intensity along the axon of control cells, the 4 h-treated cells show a strong increase in

punctuate fluorescence of TfR-GFP. It is remarkable that most peaks in the control cell are lower than the minimum brightness of the treated cell. The maxima of the treated axon are up to 6-fold higher than those of the untreated control. Staining living cells with an antibody to detect TfR that reached the cell surface showed a comparable increase, demonstrating that TfR vesicles fuse with the plasma membrane when they can enter the axon.

Quantitative analysis of 28 cells shows that KIF13B tail linked to KIF5C motor domain significantly increased both total TfR and cell surface TfR in the axon (Figure 4C). While there were treated cells with TfR levels that fell within the control range, in most cases there was a marked increase in axonal TfR. This result is consistent with the live cell assays, in which axonal transport increases were seen in only about 55%-65% of cells.

These results confirm that the KIF13B split kinesin can misdirect TfR, causing it to accumulate in the axon over time. But more importantly, they show that vesicles containing dendritic proteins are capable of fusing with the plasma membrane if they enter the axon. There is no fail-safe mechanism to prevent mis-targeted vesicles from delivering dendritic proteins to the axonal surface. Thus selective dendritic transport plays a crucial role in preventing dendritically polarized proteins from reaching the incorrect cellular domain. However, we cannot rule out the possibility that axonal membrane fusion plays a part in selective transport in that insertion, retrieval and re-sorting back to the soma is more efficient than allowing a dendritic protein to reach the axon terminal and be returned or degraded.

Low-Density Lipoprotein Receptor vesicles interact with KIF1A and KIF13B

To further leverage the utility of the split-kinesin assay we used this strategy to identify the kinesins that interact with vesicles carrying a different dendritic cargo protein, Low-density Lipoprotein Receptor (LDLR). Low-density Lipoprotein Receptor is not endogenously expressed by hippocampal neurons, but when expressed it is highly polarized to the dendritic surface and vesicles labeled by GFP-tagged LDLR undergo dendrite-selective transport [52, 96]. TfR and LDLR are thought to leave the Golgi in the same vesicles, based on analysis of their sorting signals, but their endocytic recycling pathways diverge [52, 108, 109].

We expressed GFP-LDLR and KIF5C^{560}-FKBP with the same series of truncated kinesin tails, and then looked for a change in trafficking following addition of linker drug (Figure 5). Representative kymographs from this series of experiments confirm the extremely high specificity of this experimental approach (Figure 5). Only cells expressing KIF1A and KIF13B tails showed the characteristic increase of axonal transport that signifies a tail-vesicle interaction (Figure 5B, F). The remaining kinesins that were tested, KIF1Bα, KIF1Bβ, KIF13A, KIF21B, KIF17, and KIF5C failed to cause a change in transport (Figure 5C-E, G-I). Table 1 summarizes the results of testing each FRB-tail in 8 to 15 cells. The data show that all cells expressing KIF1A (8 of 8) and 72% of cells expressing KIF13B were positive; they showed a stream of LDLR vesicles entering the axon when the linker drug was added. See figure 7B for

quantification. One of ten cells expressing the KIF13A tail was positive. All of the remaining tails yielded exclusively negative results. These data further implicate Kinesin-3 family members in the transport of vesicles carrying dendritically polarized proteins.

Quantification of these results based on kymograph analyses are shown in Figure 6B. A statistically significant increase in the number of anterograde movements of LDLR vesicles was observed in cells expressing KIF1A and KIF13B tails. No significant changes in transport were observed in cells expressing other tails. These results again document the selectivity of this assay.

Because vesicles labeled by GFP-LDLR, but not TfR-GFP interact with KIF1A and the reverse is true for KIF13A, these two pools of vesicles cannot be identical. Since TfR and LDLR differ in some aspects of their endosomal recycling it is possible that this is reflected in the differential binding observed with KIF1A and KIF13A. However, a subpopulation of endosomes contains both TfR and LDLR and this population may be moved by KIF13B [110].

Discussion

In the experiments presented here we introduce a new method to identify kinesin-vesicle interactions in living cells and use it to identify three Kinesin-3 family members that bind to vesicles containing dendritically polarized proteins. The split kinesin assay reveals the high degree of selectivity that governs kinesin-vesicle interactions. For example, KIF1A binds vesicles labeled by LDLR-GFP and pulls them into the axon following split kinesin assembly, but the closely related family members KIF1Bα and KIF1Bβ yielded entirely negative results in this assay. Likewise the highly homologous kinesins KIF13A and KIF13B exhibit distinct vesicle binding specificities. The split kinesin assay provides a unique perspective on kinesin-cargo selectivity because it can be used to probe interactions between a defined vesicle population and a broad range of kinesins. Defining how specific motors are selectively linked to their vesicular cargoes remains a challenging problem [111] and current methods seldom allow an unbiased evaluation of interactions between a given vesicle population and all relevant kinesin family members.

Our results identify three kinesins that interact specifically with dendritically polarized vesicles populations, but they do not rule out the possibility that other kinesins participate in the transport of the tested cargo vesicles. Nor do they imply that the dendritic vesicle transport is mediated exclusively by kinesins. Disrupting dynein- and myosin-mediated transport enables dendritic proteins to enter the axon, much as disrupting the selectivity of kinesin-mediated transport

causes TfR to mis-localize to the axon [57, 58]. Considerable evidence indicates that multiple motors bind the same vesicle simultaneously under circumstances that permit their activity to be coordinately regulated [111]. On the other hand, the identification of Kinesin-3 family members that bind specifically to both populations of dendritic vesicles examined in this study *does* represent a significant challenge for models that propose kinesins play no role in dendrite-selective vesicle transport [57].

The Kinesin-3 family and dendrite selective vesicle transport

Our results, summarized in Figure 6, show that KIF13A and KIF13B bind to dendritically polarized vesicles containing TfR and that KIF1A and KIF13B bind to dendritic vesicles containing LDLR. The association of KIF1A with dendritic vesicles was not unexpected. Previous work has shown that KIF1A associates with dendritically polarized AMPA receptors through the liprin-α cargo adaptor [49, 112]. KIF1A is also a principal motor for transporting synaptic vesicle proteins in the axon and for transporting secretory granules in the axon and dendrites [105, 112]. In accordance with these previous studies, we found that GFP-tagged KIF1A labels vesicles in both the axon and dendrites (Figure 1A).

By comparison, the observation that KIF13A and KIF13B selectively bind dendritic vesicles was quite surprising. Neither of these kinesins has previously been implicated in dendritic transport. Little is known about cargoes carried by KIF13A, but it has been shown to interact with the AP-1 adaptor complex and to

play a role in the transport of Mannose-6-Phosphate receptor [40]. KIF13B has previously been implicated in the transport of PIP_3 to growing axons, but it also interacts with an isoform of SAP97, which largely localizes to postsynaptic sites in dendrites [66, 79, 113]. GFP-tagged KIF13B labels vesicles that are dendritically polarized—indeed, it is the only kinesin we have found that is exclusively associated with dendritic vesicles. Thus the main role of KIF13B may be as a motor for dendritic transport.

Mechanisms underlying dendrite-selective transport

Two principal models have been proposed to explain the role of kinesins in selective dendritic transport. The “smart motor” hypothesis posits the existence of a subset of kinesins that can distinguish structural differences between axonal and dendritic microtubules and translocate preferentially to one or the other domain [6, 98]. Binding of a vesicle to a smart dendritic kinesin would of itself ensure its dendrite-selective transport. The alternative “cargo steering” or “cargo regulatory” hypothesis proposes that a single kinesin may mediate either axon- or dendrite-directed transport, depending on whether it interacts with a vesicle containing axonal or dendritic proteins [36]. In support of this hypothesis, both KIF5 and KIF1A, which play a well-documented role in axonal transport, also interact with dendritically polarized glutamate receptors [36, 49].

We show here that KIF1A interacts with dendritically polarized LDLR vesicles and confirm that this same kinesin also associates with secretory

granules, which are transported into the axon (Figure 6). These results are inconsistent with the smart motor hypothesis. KIF13A, which is present on dendrite-selective TfR-containing vesicles, also associates with vesicles that are transported into the axon (Figure 1). However, the cargo contained in these vesicles has not yet been identified. A recent study in *Drosophila* showed that Khc, a member of the Kinesin-1 family, participates in the dendrite selective transport of some cargoes and mediates axonal transport of other cargoes [98]. KIF13B has some of the characteristics of a smart motor—GFP-tagged KIF13B is associated with vesicles that are largely confined to the somatodendritic domain. However, the motor domain of KIF13B is capable of walking on axonal microtubules, which suggests that other factors also regulate the translocation selectivity of this kinesin [101].

The axon initial segment marks the boundary between the axonal and somatodendritic domains and vesicles carrying dendritic proteins do not proceed beyond this boundary [3, 6]. Several models suggest that the transport of dendritic vesicles is inhibited when they reach this region of the cell. Song et al.. show that the initial segment contains a dense actin network and propose that the kinesins that carry dendritic vesicles lack the strength to traverse this network [47]. Lewis et al. show that myosins are essential for selective dendritic transport and propose that myosins bound to dendritic vesicles are activated in the initial segment, anchoring the vesicles to actin filaments and preventing them from being transported into the axon along microtubules [58]. Regulation within the initial segment could also alter the balance between kinesin and dynein-based

motility. Finally, entry into the axon may depend on whether or not a kinesin walks efficiently on the unique subset of microtubules that traverse the initial segment [97, 102, 114]. The results presented here impact all of these models. Any model of selective transport must explain how kinesins are prevented from entering the axon when they carry a dendritic vesicle but are perfectly capable of passing through the initial segment when they carry an axonal vesicle. Elucidating the molecular mechanisms that enable cargo binding to regulate kinesin translocation will be essential in refining our understanding of selective vesicle transport.

Detecting kinesin-vesicle interactions using split kinesins

In principle, the split kinesin assay is capable of identifying the kinesins that interact with any fluorescently labeled vesicle population in any cell type, so long as linking the vesicles to a constitutively active motor domain will result in a distinctive pattern of transport. One advantage of this method is the high specificity with which it detects kinesin-vesicle interactions. Of the sixteen possible tail-vesicle combinations tested in our screen, only four combinations yielded a positive readout. Interactions that were scored as positive caused a pronounced increase in transport in 55% to 90% of recorded cells. This is a striking result, since there was essentially no axonal transport of these vesicles in the same cells prior to assembling the split kinesins. Conceivably, over-expression of a kinesin tail could enable it to bind vesicles non-specifically, but the selectivity of the kinesin binding observed in these experiments suggests that

this is unlikely. The likelihood of false negatives in this assay is more difficult to evaluate. Because the expressed KIF5C motor domain is constitutively active, it builds up in the axon tips of the expressing cells and only a small fraction of the total motor is able to attach to vesicles in the soma. Therefore, low expression of the motor domain could lead to a negative result. Similarly, kinesin tail expression must be adequate to enable enough copies to bind to the vesicles, even in the presence of endogenous kinesins, to produce a clear change in transport once the split kinesin is assembled.

Until now, the tools available to identify the kinesins that transport specific vesicles have been limited. Most prominently, siRNA knockdown and dominant negative strategies have been applied, but both approaches suffer from significant limitations [18, 106]. Both require comparatively long expression times, which can lead to secondary effects, and the inhibition observed in vesicle transport assays can be modest, requiring statistical analysis of recordings from many cells [105]. Both strategies can lead to false negative results when multiple kinesins transport the same vesicle population. By contrast, the split kinesin method works equally well regardless of the number of different kinesins associated with a given vesicle and the change in transport that occurs when the split kinesin is assembled is so obvious that it can easily be seen in recordings from individual cells.

Adaptations of this method are likely to have a broad range of applications. For example, it should be possible to evaluate vesicle interactions with myosins or dynein-associated proteins by tagging them with the FRB

domain and linking them to an appropriate FKBP-tagged kinesin motor domain. The assay could also be used to determine whether two proteins co-localize on the same vesicle. By fusing an FRB to one of the proteins in question, that protein could be linked to a FKBP-tagged kinesin motor domain, leading to vesicle transport when the linker drug is added. The coordinated movement of both proteins would provide compelling evidence for co-localization.

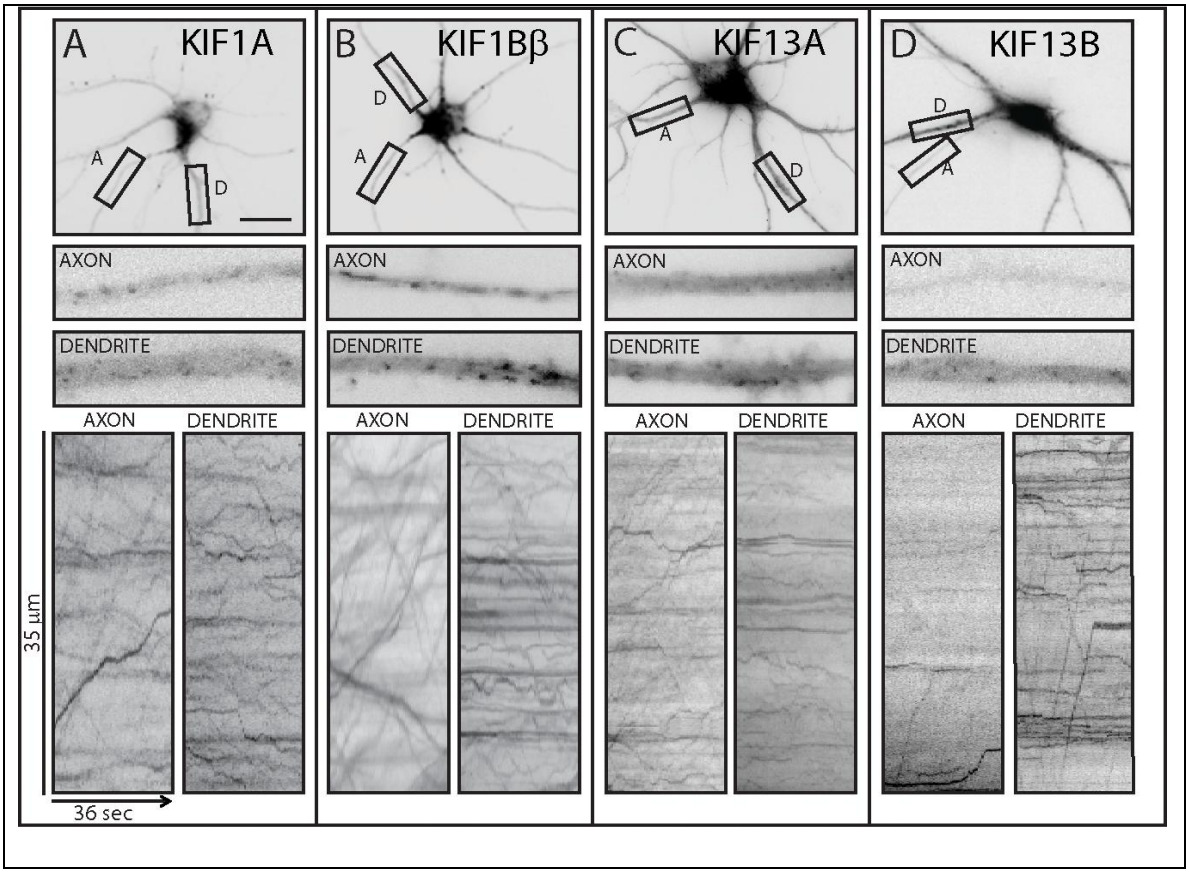

A
KIF1A
A
D
B
KIF1Bβ
D
A
C
KIF13A
A
D
D
KIF13B
D
A
AXON
DENDRITE
AXON
DENDRITE
AXON
DENDRITE
AXON
DENDRITE
AXON
DENDRITE
AXON
DENDRITE
AXON
DENDRITE
35 µm
36 sec

Figure 1. Vesicles labeled by expressing full-length GFP-tagged Kinesin-3 motors

Fluorescently labeled full length kinesins were expressed in cultured hippocampal neurons to label vesicles. (A-D) Representative images of axons and dendrites of hippocampal neurons expressing GFP-tagged KIF1A, KIF1Bβ, KIF13A and KIF13B, respectively. Boxed regions are shown at high magnification below. Kymographs illustrating vesicle movements in the boxed region of axons and dendrites show that for KIF1A, KIF1Bβ, KIF13A there is bi-directional transport of labeled vesicles in both axons and dendrites, but KIF13B vesicles were largely polarized to the dendrites. The contrast has been inverted to make fluorescent objects appear dark on a light background. Scale bar: 20 μm.

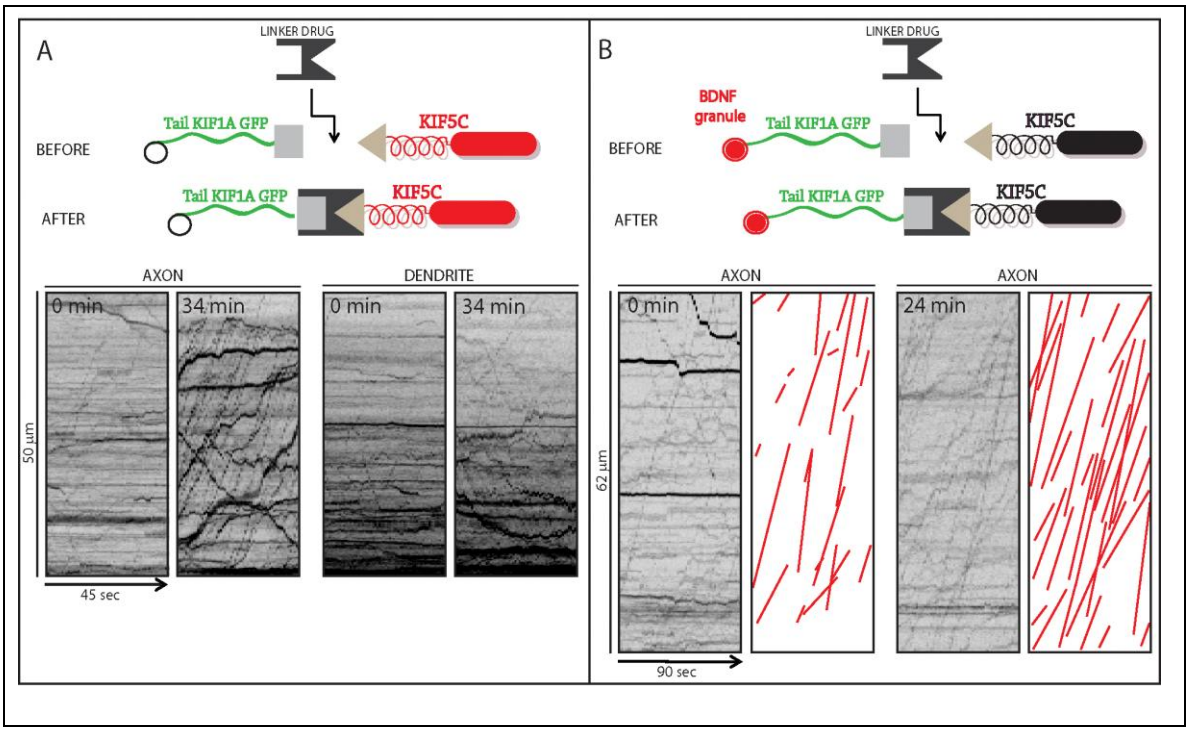

A
LINKER DRUG
BEFORE
Tail KIF1A GFP
KIF5C
AFTER
Tail KIF1A GFP
KIF5C
AXON
DENDRITE
0 min
34 min
0 min
34 min
50 µm
45 sec
B
LINKER DRUG
BDNF granule
BEFORE
Tail KIF1A GFP
KIF5C
AFTER
Tail KIF1A GFP
KIF5C
AXON
AXON
0 min
24 min
62 µm
90 sec

Figure 2. A distinctive change in the pattern of vesicle transport is induced by linking a tail-vesicle complex to an active axon-selective motor domain

Hippocampal neurons were transfected with a split kinesin consisting of a GFP-labeled KIF1A tail and a KIF5C motor domain. Addition of the linker drug resulted in an increase of vesicles moving into the axon.

(A) Illustration of the chemical assembly of the KIF1A-GFP split kinesin. The constructs were expressed in hippocampal neurons for 18 hours and live imaging experiments were performed to evaluate the transport of KIF1A-labeled vesicles after assembly of the split kinesin. After 25 min of treatment with 1 μM of linker drug (AP 21967), there was a large increase in the long range anterograde transport of KIF1A labeled vesicles (positively sloped lines) in the axon, but not in the dendrites. See chapter 3 supplemental movie 1.

(B) Ilustration of the chemical assembly of the KIF1A split kinesin and its interaction with BDNF-containing secretory granules, a known KIF1A cargo. The kymographs show the transport of BDNF vesicles in the axon before and 24 min after addition of the linker drug. The traced lines showing all anterograde events (red) highlight the increase in long range transport.

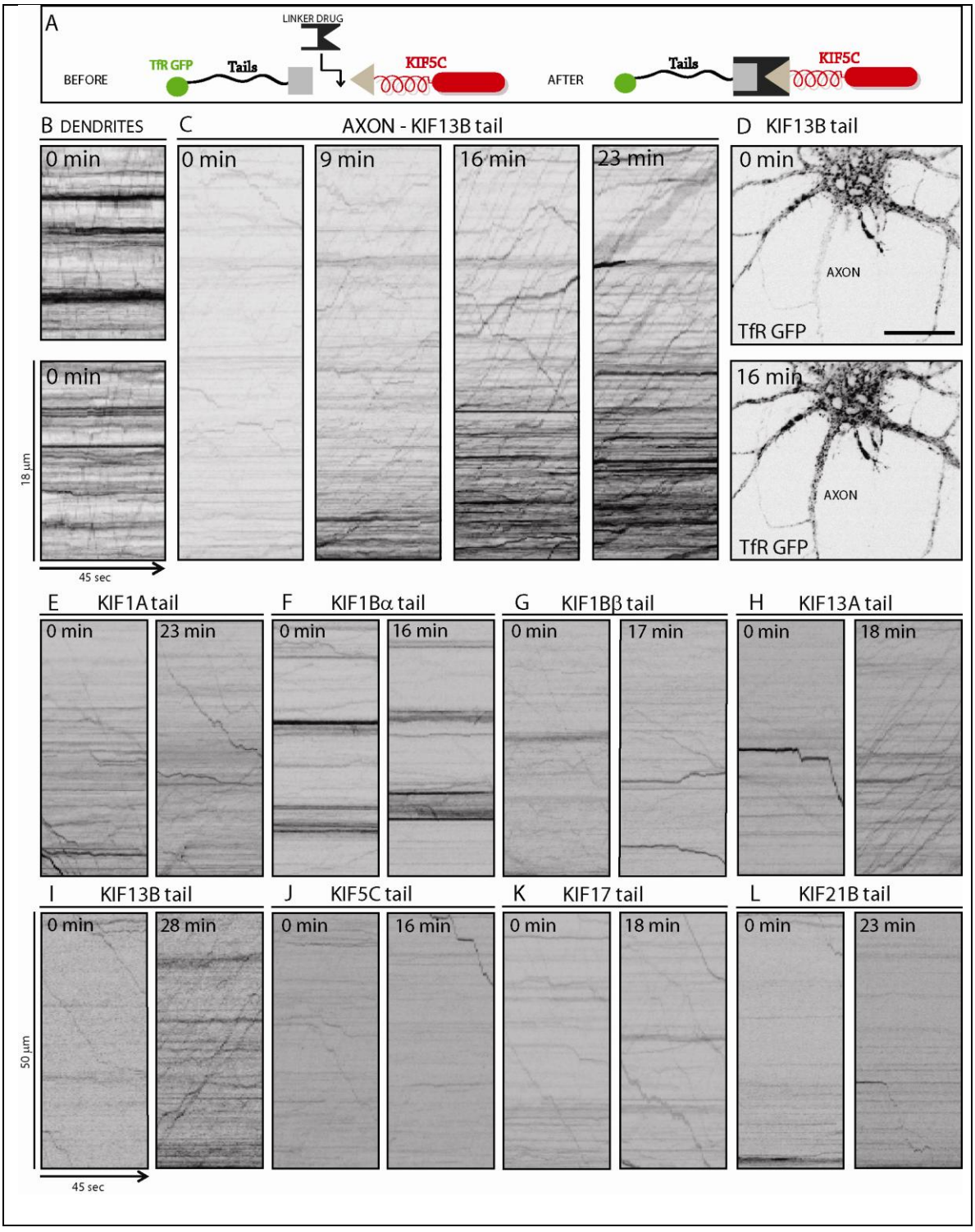

A
LINKER DRUG
BEFORE
TfR GFP
Tails
KIF5C
AFTER
Tails
KIF5C
B DENDRITES
C
AXON - KIF13B tail
D KIF13B tail
0 min
0 min
9 min
16 min
23 min
0 min
AXON
TfR GFP
0 min
16 min
AXON
TfR GFP
18 µm
45 sec
E KIF1A tail
F KIF1Bα tail
G KIF1Bβ tail
H KIF13A tail
0 min
23 min
0 min
16 min
0 min
17 min
0 min
18 min
I KIF13B tail
J KIF5C tail
K KIF17 tail
L KIF21B tail
0 min
28 min
0 min
16 min
0 min
18 min
0 min
23 min
50 µm
45 sec

Figure 3. KIF13A and KIF13B bind Transferrin Receptor (TfR) vesicles

Cells expressing fluorescent TfR and a variety of split kinesins were recorded. Movement of TfR vesicles in the axon was recorded before and after assembly of the split kinesin.

(A) Illustration of the chemical assembly of different split kinesins that were co-expressed with TfR-GFP to label vesicles.

(B) Kymographs showing the transport of TfR vesicles before assembly of the split kinesin in two different dendrites.

(C) Kymographs showing the transport of TfR vesicles mediated by assembled KIF13B split kinesin over time (0, 9, 16 or 23 min after the addition of 1 μM of linker drug). Note the remarkable increase in the long range anterograde movement of TfR vesicles after few minutes of treatment.

(D) Images showing the cell body and proximal axon of the neuron imaged immediately before (0 min) and after 16 min of treatment with linker drug. Note the increase in intensity of TfR-GFP in the axon after 16 min.

(E-L) Different split kinesin constructs were expressed in hippocampal neurons for 18 hours and live imaging experiments were performed. The kymographs show the transport of TfR vesicles in the axon before and 14-28 min after addition of the linker drug. There is no change in the overall transport of TfR vesicles when KIF1A, KIF1Bα, KIF1Bβ, KIF5C, KIF17, or KIF21B tails were expressed. However, there was a large increase in the long range anterograde transport events of TfR vesicles when KIF13A (H) or KIF13B (I) tails were used. Scale bar: 20 μm. See chapter 3 supplemental movie 2.

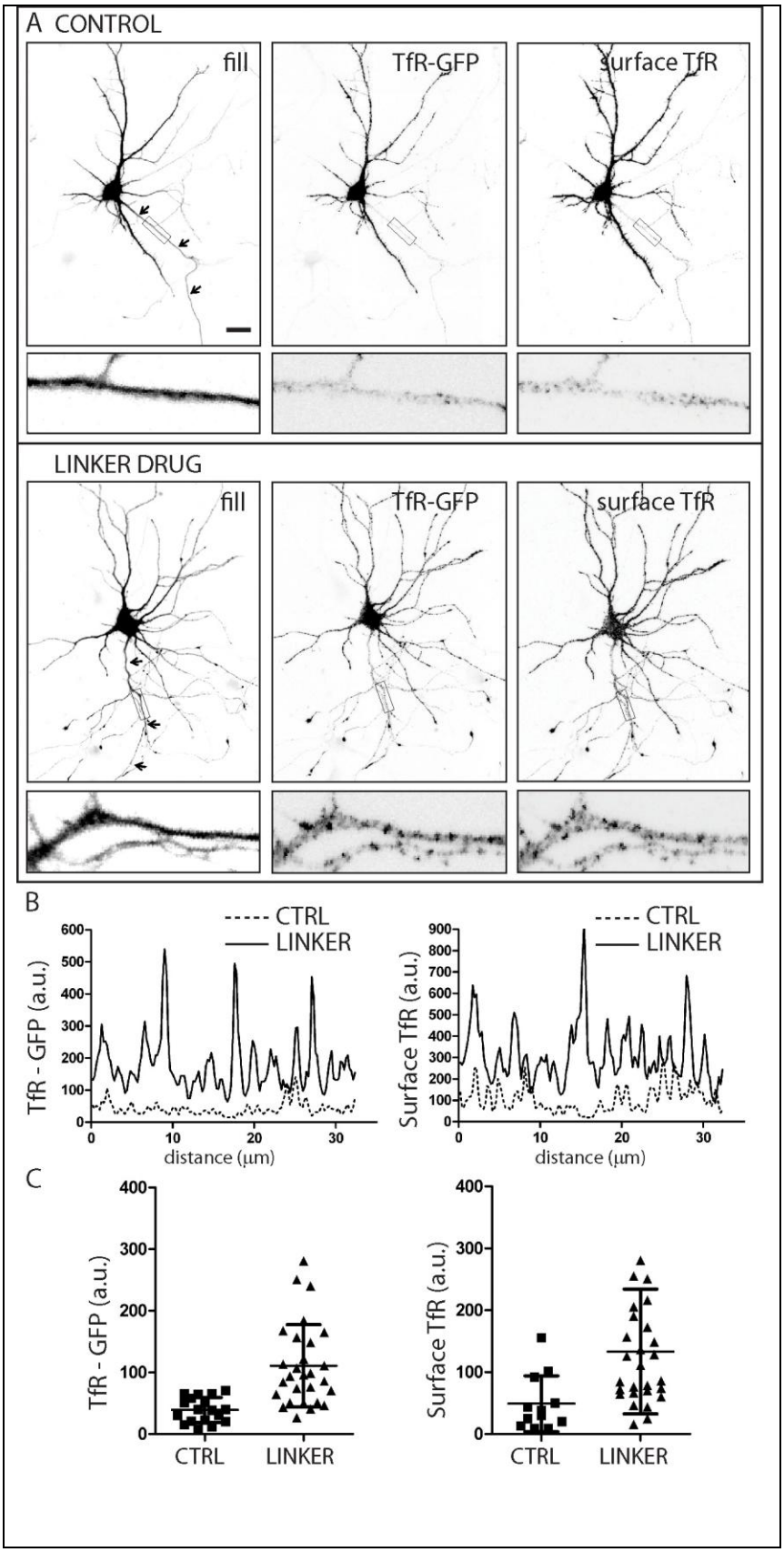

A CONTROL
fill
TfR-GFP
surface TfR
LINKER DRUG
fill
TfR-GFP
surface TfR
B
CTRL
LINKER
TfR - GFP (a.u.)
distance (μm)
CTRL
LINKER
Surface TfR (a.u.)
distance (μm)
C
TfR - GFP (a.u.)
CTRL
LINKER
Surface TfR (a.u.)
CTRL
LINKER

Figure 4. Accumulation of mis-directed TfR in the axon

Cells expressing TfR-GFP, a KIF13B split kinesin, and soluble eBFP2 to fill the entire axonal and dendritic arbor were incubated with the linker drug for 4 h. To detect the total amount of GFP in the axon, the GFP fluorescence was assessed. To detect the TFR present on the cell surface, live-staining was performed using a monoclonal antibody against GFP.

(A) Representative control and treated cells showing eBFP2 fill, TfR-GFP, and live TfR surface staining. Boxed regions of the axon are shown at high magnification below. Scale bar: 20 µm.

(B) Line scans of the boxed regions of axon show total TfR-GFP or TfR that reached the cell surface.

(C) Dot plots showing the average fluorescence intensity of axonal regions. Regions were chosen in the proximal and medial axon of every cell. The average intensity of both line scans is shown. Each point represents one cell. Notice the pronounced increase in TfR in the axon 4 h after addition of the linker drug.

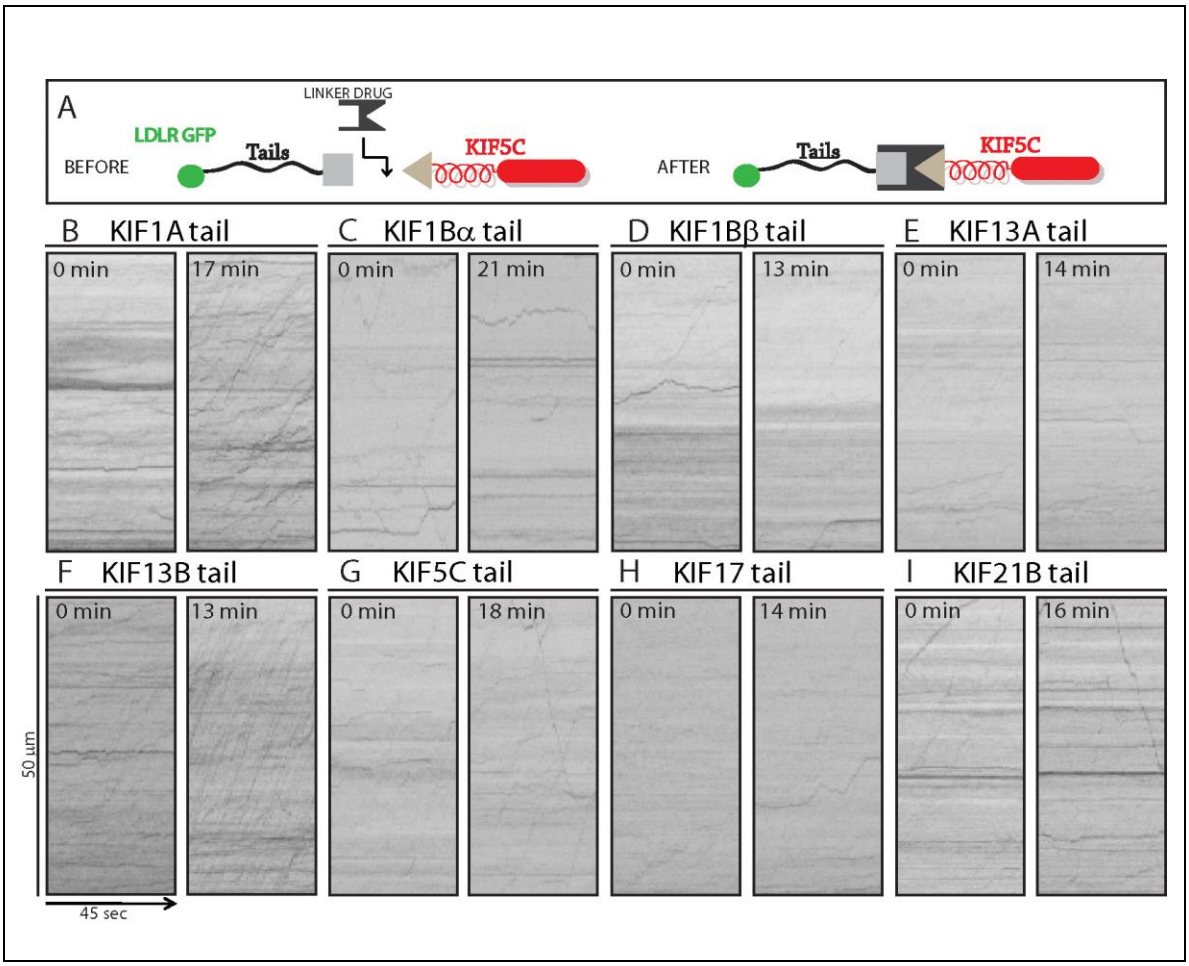

A
LINKER DRUG
LDLR GFP
Tails
KIF5C
BEFORE
AFTER
Tails
KIF5C
B KIF1A tail
0 min
17 min
C KIF1Bα tail
0 min
21 min
D KIF1Bβ tail
0 min
13 min
E KIF13A tail
0 min
14 min
F KIF13B tail
0 min
13 min
G KIF5C tail
0 min
18 min
H KIF17 tail
0 min
14 min
I KIF21B tail
0 min
16 min
50 um
45 sec

Figure 5. KIF1A and KIF13B bind Low-Density Lipoprotein Receptor (LDLR) vesicles

GFP-LDLR was expressed with different split-kinesin constructs.

(A) Illustration showing the chemical linking of split kinesin constructs that were co-expressed with GFP-LDLR. Movement of LDLR vesicles in the axon was recorded before and after split kinesin assembly.

(B-I) Different split kinesins were expressed in hippocampal neurons for 18 h and live imaging experiments were performed. The kymographs show the transport of LDLR vesicles in the axon before and 13-21 minutes after the addition of linker drug. There is no change in the overall transport of LDLR vesicles when KIF1Bα, KIF1Bβ, KIF13A, KIF5C, KIF17, KIF21B split kinesins were used. However, there is a large increase in the long range anterograde LDLR moving vesicles when KIF1A (B) or KIF13B (F) were expressed.

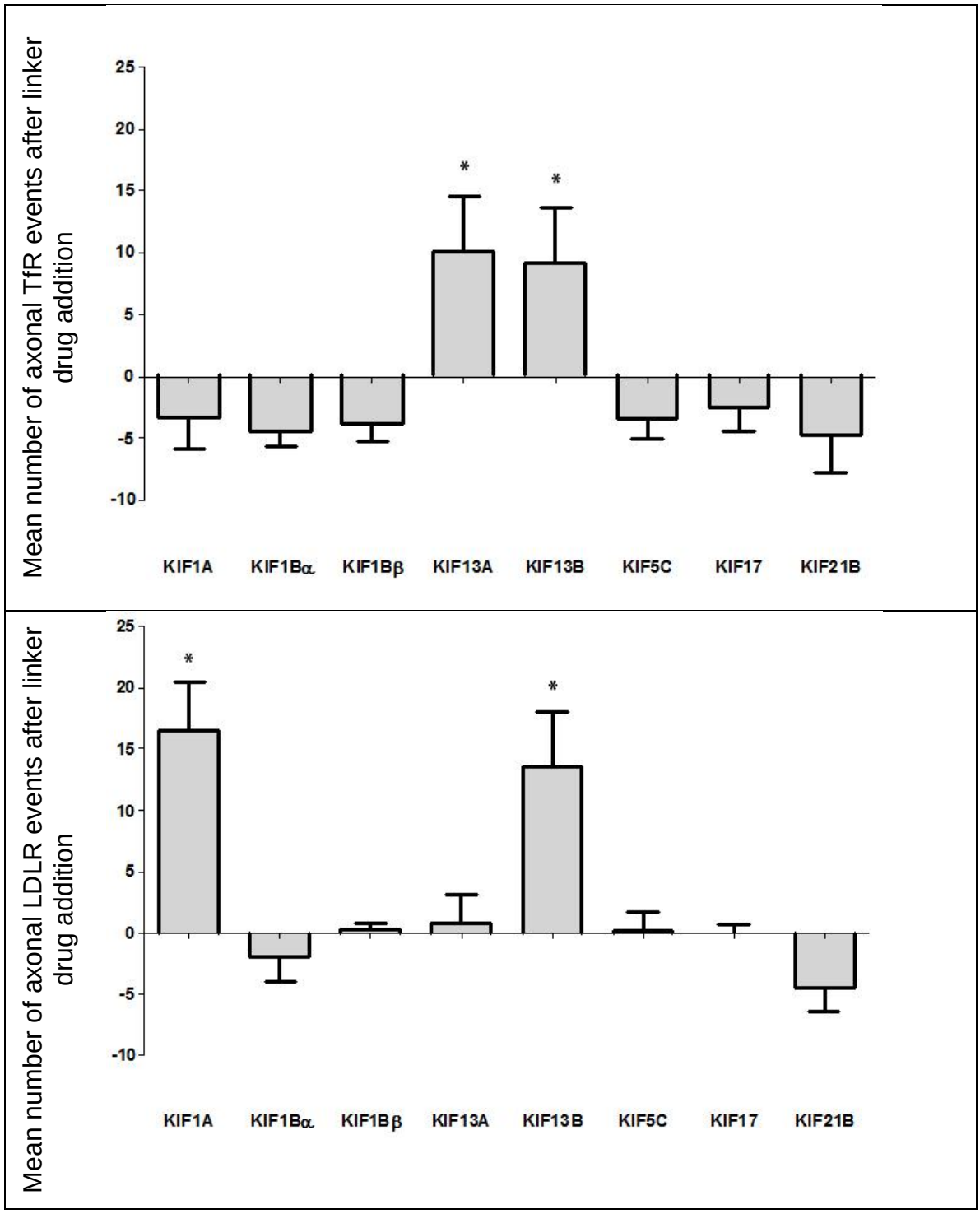

Mean number of axonal TfR events after linker drug addition
25
20
15
10
5
0
-5
-10
*
*
KIF1A
KIF1Bα
KIF1Bβ
KIF13A
KIF13B
KIF5C
KIF17
KIF21B
Mean number of axonal LDLR events after linker drug addition
25
20
15
10
5
0
-5
-10
*
*
KIF1A
KIF1Bα
KIF1Bβ
KIF13A
KIF13B
KIF5C
KIF17
KIF21B

Figure 6. Quantification of transport events induced by the split-kinesin assay. Anterograde events were traced from kymographs for each cell before and 15-30minutes after linker drug addition. A, the average change in axonal TfR events for each kinesin tail co-expressed. n=8-16 cells. B, The average change in axonal LDLR events for each kinesin tail co-expressed. n=8-11 cells. Kymographs were quantified blind. Wilcoxon signed rank test ($p < 0.01$)

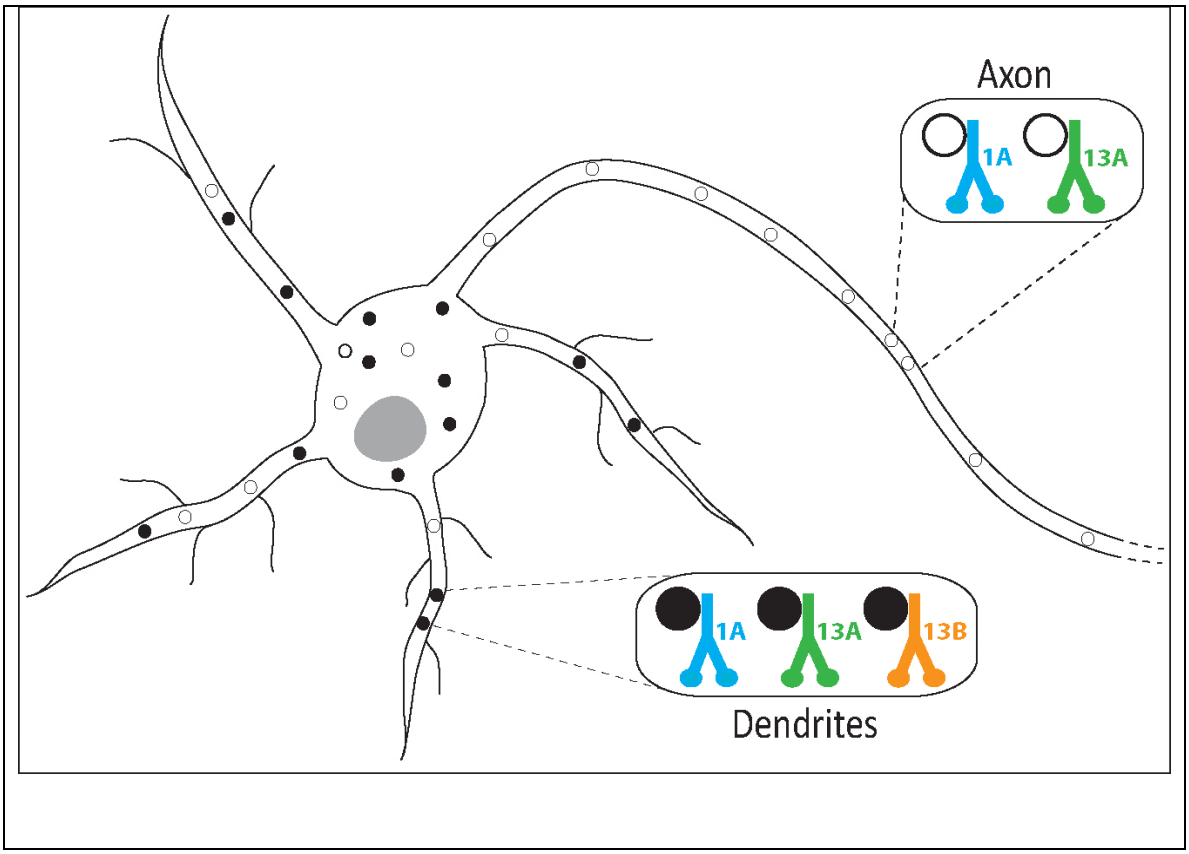

Axon
1A
13A
1A
13A
13B
Dendrites

Figure 7. Summary diagram illustrating the role of Kinesin-3s in polarized transport. KIF1A, KIF13A, and KIF13B associate with vesicles containing dendritic proteins (filled circles) that are selectively transported in dendrites. KIF13A and KIF13B are found on TfR vesicles (Figure 3), while KIF1A and KIF13B are found on LDLR vesicles. Two of the three kinesins that participate in selective dendritic transport are also involved in the transport of axonal vesicles (open circles).

Experimental Procedures

Cell Culture

Primary hippocampal cultures were prepared from embryonic day 18 rats and maintained in MEM with N2 supplements, and transfected as described previously [94]. Constructs were transfected into Stage 4 hippocampal neurons (8-12 days in culture) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and expressed for 8-18 h before imaging.

Constructs and Imaging

Full-length kinesins are functional dimers. The motor domain that interacts with microtubules is located at the N-terminus, directly followed by a neck-linker and the coiled-coil that facilitates dimerization [81] . The motor domain used in all experiments is $KIF5C^{560}$-TdTM-FKBP. $KIF5C^{560}$ homo-dimerizes and forms a constitutively active motor that moves to the plus-end of microtubules [54]. Kinesin tails were all engineered following the same strategy: The N-terminal was removed and replaced with an FRB-3myc domain. All constructs used the pCAG expression vector.

The linkage of tail to the active motor domain was induced by treating cells expressing motor and tail constructs with AP 21967, a rapamycin analog [57, 115] Movies of vesicle transport (60-90 frames, 600-750 ms/frame) were collected using a spinning-disk confocal microscope and time-distance “kymographs” of vesicle motion were generated using MetaMorph software (Molecular Devices, Sunnyvale, CA).

During imaging, cells were maintained at 32–34°C in a heated chamber (Warner Instruments, Hamden, CT) containing Hibernate E without phenol red (BrainBits, Springfield, IL) supplemented with B27 (Invitrogen, Carlsbad, CA). Objectives were warmed to 34°C (Bioptechs Inc.). Live images were acquired on a Nikon TE2000 with a spinning-disk, confocal-head (Solamere Technology Group; Yokogawa CSU10) with an Orca-ER CCD camera (Hamamatsu Photonics Co., Ltd). A Nikon Plan-Apo 60x 1.45 NA objective was used to acquire movies. During movie acquisition Z-axis movement was controlled by Nikon TE2000 Perfect Focus. Movies were acquired by streaming 60-90 frames (600-750ms/frame) before drug treatment and at 10-15min intervals after drug treatment. Movies for figure 1 were acquired on a Leica DM RXA (Leica Microsystems, Wetzlar, Germany) with a Micromax CCD camera (Princeton Instruments) using a 63x Plan-Apo 1.32 objective. Cells were maintained as above but using imaging media consisting of HBSS with Ca^{2+}, Mg^{2+}, and 10mM HEPES (Gibco) with 0.6% glucose. MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to drive microscopes and analyze movies.

In all figures, the contrast has been reversed so that fluorescent objects appear dark on a light background.

TfR-GFP and GFP-LDLR sequences were used as previously described, but expressed with the pCAG expression vector and lipofectamine [6, 52, 96].

Chapter 4: Summary, discussion and future directions

Summary and discussion

This dissertation began with the observation that GFP-KIF13B-labeled vesicles traffic exclusively in dendrites. This observation led to the hypothesis that KIF13B is a dendrite-selective kinesin, and that the motor domain was responsible for its selectivity. Evidence from the truncated motor assay supported this hypothesis, in that KIF13B was one of only a small number of kinesins with a motor domain capable of accumulating in the dendrites [23]. To explore this hypothesis I tested the ability of KIF13B to transport cargo into the axon compared with the dendrites. Using the peroxisome assay, I demonstrated that the motor domain transports peroxisomes equally well into both the axons and dendrites. This result suggests regulation of KIF13B-labeled dendritic vesicles cannot be solely attributed to the motor domain. To test whether another region within KIF13B is responsible for dendrite-selective transport I created various truncation and deletion constructs which I then used in the peroxisome assay. These experiments did not uncover a region responsible for dendrite-selective transport but did confirm that the MBS domain within the tail region likely contributes to the autoinhibition of KIF13B. In the future directions section, I will discuss how this regulation may contribute to KIF13B-mediated dendrite-selective transport.

The technique of using peroxisomes as an artificial cargo was rather novel and previous studies demonstrated dynein and conventional kinesin were able to transport peroxisomes. However this assay may not be useful for studying a kinesin that requires forced to dimerization to translocate. The Kinesin-3 family,

unlike the Kinesin-1 family, has been suggested to transport cargo as both a dimeric and a monomeric kinesin [64, 71, 81]. We were uncertain if the peroxisome assay could distinguish monomeric and dimeric kinesin-based transport. To address whether a monomeric kinesin can function in this assay I tested the known monomer and dimer forms of KIF1A and found that a monomeric KIF1A can indeed transport peroxisomes – though at a slower velocity than the dimer. Accordingly, this assay has limited use for testing the motor domain, unless the assay can be modified to require dimerization (discussed in chapter 2). Nevertheless, my results using the peroxisome experiments suggest the motor domain is not sufficient for dendrite-selective transport. Instead, another factor (either a soluble protein or another protein attached to the vesicle) may play an essential role in restricting KIF13B-labeled vesicles from entering the axon.

To understand the underlying mechanism that is responsible for kinesin-mediated dendrite-selective transport, a first step is to identify the kinesins that transport dendritic cargoes. In the introduction I discussed examples where the interaction of specific kinesins with certain axonal and dendritic cargoes was proposed. While these studies aided our understanding of kinesin-cargo relationships, they did not help in understanding selective transport, because none provided direct evidence for a kinesin transporting a specific cargo. Furthermore, the examples did not definitively show the kinesin and cargo were bound to the same vesicles within the cell. Thus, the mechanism for dendrite-selective transport remains an open question.

In the third data chapter I presented a novel technique that I, along with collaborators in the lab, developed to identify the kinesins responsible for transporting dendritic vesicles. Once the kinesin-cargo interaction is identified, experiments can be done to dissect out a mechanism for transport selectivity. This technique is not limited to identifying kinesins that interact with dendritic cargos. Variations of this assay can be used to identify the kinesins that interact with axonal vesicles. In this case other motor domains can be used to reverse or stop axonal transport. The KIF5C motor domain could be substituted with a Dynein or KIFC2 motor domain which transport to the minus-ends of microtubules. If acutely attached to a cargo moving in the axon, these motor domains would be expected to stop the cargo and return it to the soma. Similarly, a kinesin rigor mutant binds ATP but cannot hydrolyze it, thereby locking the motor domain to the microtubule. Using a rigor mutant motor domain in the split-kinesin assay would result in cargo vesicles becoming anchored to the microtubules. Attaching the tail domain of kinesins to any of these other motor domains would result in the altered transport of the candidate vesicle population. Together with my collaborators, I am investigating these additional possibilities.

The split-kinesin technique may be used in other ways besides identifying the kinesin tails that interact with vesicles. As previously discussed other methods for testing protein interactions or overlapping patterns of expression suffer from some limitations. The split kinesin technique could be used to identify if two proteins bind to each other in living cells or co-localize in the same compartment. In this experiment, one of the proteins of interest (protein A) is

labeled with a fluorescent protein and the other (protein B) is fused to an FRB domain. Cells are then co-transfected with these constructs, along with a constitutively active motor domain fused to an FKBP domain. Upon addition of the linker drug the motor domain will bind the FRB protein and transport it. If protein A and protein B are in the same vesicle/compartment or bound to each other, then they both will move together. In fibroblasts, the protein A - protein B complex will move to the cell periphery since the majority of the microtubule plus-ends are located at the cell periphery.

Another variation of this split-kinesin technique could be used to test the myosin model for dendrite-selective transport, outlined in the introduction. The myosin model states that myosinVa is present on dendritic vesicles and prevents them from entering the axon by interacting with actin filaments in the axon initial segment. However, data presented in chapter three shows that dendritic vesicles can be driven into the axon by linking them to an active kinesin motor domain. The myosin model would have predicted that these dendritic vesicles should not enter the axon. To explore the myosin model further using the split kinesin technique, it may be possible to link the myosinVa motor domain to a kinesin tail attached to axonal cargoes. The myosin model would predict these axonal vesicles would no longer enter the axon.

The following section outlines a few experiments to further investigate the involvement of KIF13B in dendrite-selective transport.

Future Directions

Overall, my thesis is focused toward identifying a mechanism for kinesin-based dendrite-selective transport. In chapter three, I presented data identifying which kinesins bind to dendritic vesicles; however, a number of additional questions remain regarding how this interaction is regulated to restrict dendritic vesicles from entering the axon. In this section I will discuss experiments that follow up on my dissertation.

Investigating the KIF13B linkage to TfR vesicles

For the first experiment, I would investigate how the KIF13B tail binds to the TfR vesicle and test whether this interaction could be involved in dendrite-selective transport. It could be that the KIF13B-vesicle interaction is somehow disrupted near the axon initial segment to prevent dendritic vesicles from entering the axon. Some kinesin-cargo interactions have been shown to be regulated by phosphorylation or Ca++ [116-119]. A simple mechanism to detach the kinesin from its cargo could contribute to dendrite-selective transport.

To test if the regulation of the kinesin-cargo interaction contributes to selective transport, I first need to determine the region or regions within the KIF13B tail that bind to TfR-containing vesicles. To this end, I would split the tail into two regions and attach an FRB domain to each of them. Then, using the split-kinesin assay, I would test whether each region of the tail domain retains the ability to bind to TfR. Assuming one of the two regions binds to TfR vesicles, I would further dissect this region in the same manner to identify the minimal binding region. It is possible that dividing an interaction region into two parts may

prevent both portions from binding their normal proteins. Designing a construct that spans the border region would test if this region is responsible for KIF13B to interact with its cargo.

Once the minimal region of interaction is found, I would first scan the sequence for predicted phosphorylation sites, as some protein-protein interactions are known to be phosphorylation dependent. If a candidate phosphorylation site is identified then mutating this site to either a phospho-mimic (glutamate) or a non-phosphorylatable residue (alanine) can be used to address its role in cargo-binding. Using the split-kinesin assay I would test both phosphorylation mutants their ability to direct trafficking of TfR-containing vesicles. For example, if phosphorylation is necessary for the KIF13B-vesicle interaction, then the phospho-mimic would show vesicles entering the axon in the split-kinesin assay. In contrast, the non-phosphorylatable version should not mediate transport of these vesicles into the axon (Figure 1A). These results would provide support that interaction between KIF13B and the TfR vesicles is phosphorylation-dependent.

To identify proteins that bind to the minimal interaction domain of KIF13B, I would use the minimal binding region in an immunoprecipitation assay, using vesicles fractions and lysates from rat brains, as previously described [44, 49]. Vesicle fractions would be used to screen for proteins on the vesicle that interact with KIF13B, while brain lysate would be used to identify soluble proteins that interact with KIF13B. Pull-down samples would be analyzed by mass-spectrometry to obtain sequences of candidate interacting proteins. Candidates

identified in this manner would be cloned and fused to an FRB domain and then assayed in the split-kinesin assay to test whether they bind to the vesicles in a cellular environment. Neurons would be transfected with the candidate-FRB, KIF5C motor-FKBP and TfR-GFP. If the candidate protein binds to TfR-containing vesicles, then linking the KIF5C motor-FKBP to the candidate-FRB would result in an increase of axonal transport of TfR-labeled vesicles (Figure 1B). In summary, I believe identifying binding partners for KIF13B may shed light on the mechanism for kinesin-mediated dendrite-selective transport.

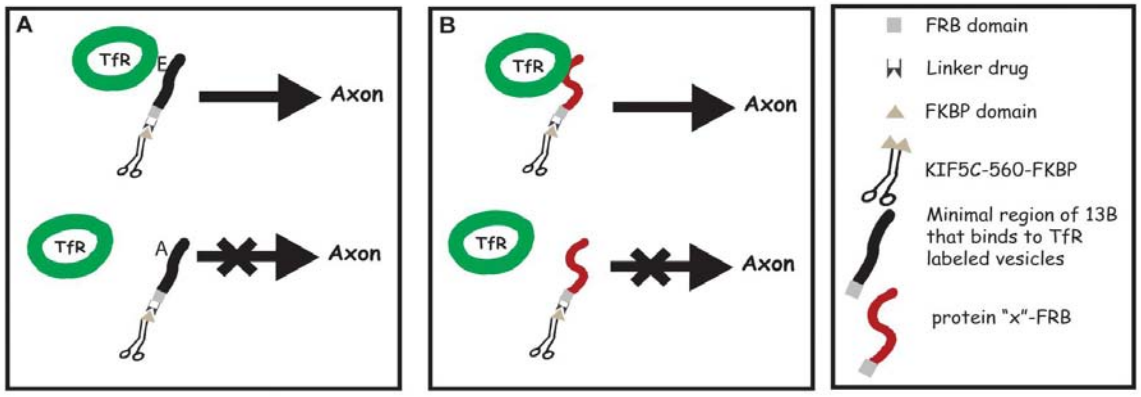


Figure 1. A. Model for phosphorylation-dependent cargo binding. Top cartoon shows the phospho-mimic mutation to the responsible residue allowing TfR-containing vesicles binding to the minimal region of KIF13B. Bottom cartoon shows the non-phosphorylatable mutation to alanine inhibits the interaction between TfR-containing vesicles and the minimal region of KIF13B. B, A test of the ability of candidate protein X's to bind to TfR-containing vesicles. A KIF5C-FKBP – FRB Protein X complex will carry TfR-containing vesicles into the axon if protein X binds to TfR-containing vesicles.

Regulation of KIF13B: Contribution of SAP97 and the MBS

In the previous section, I discussed the contribution of the kinesin-cargo interaction for regulating KIF13B-mediated dendrite-selective transport. In a

different model, I propose that the regulation of KIF13B activity contributes to its dendrite-selective transport by becoming inactive near the axon-initial segment. The well-characterized KIF13B-binding protein SAP97 has been suggested to regulate the activity of KIF13B [69]. In a microtubule-stimulated ATPase activity assay, full-length KIF13B was found to be inactive. However, in the presence of a region of SAP97, KIF13B was activated. To test if SAP97 can activate KIF13B in neurons, I would co-express SAP97 with full-length KIF13B in the peroxisome assay. In chapter 2, I presented data showing that the full-length KIF13B was unable to move peroxisomes, possibly because it is inactivated. It is conceivable that in the presence of excess SAP97, full-length KIF13B can be activated to move peroxisomes (Figure 2).

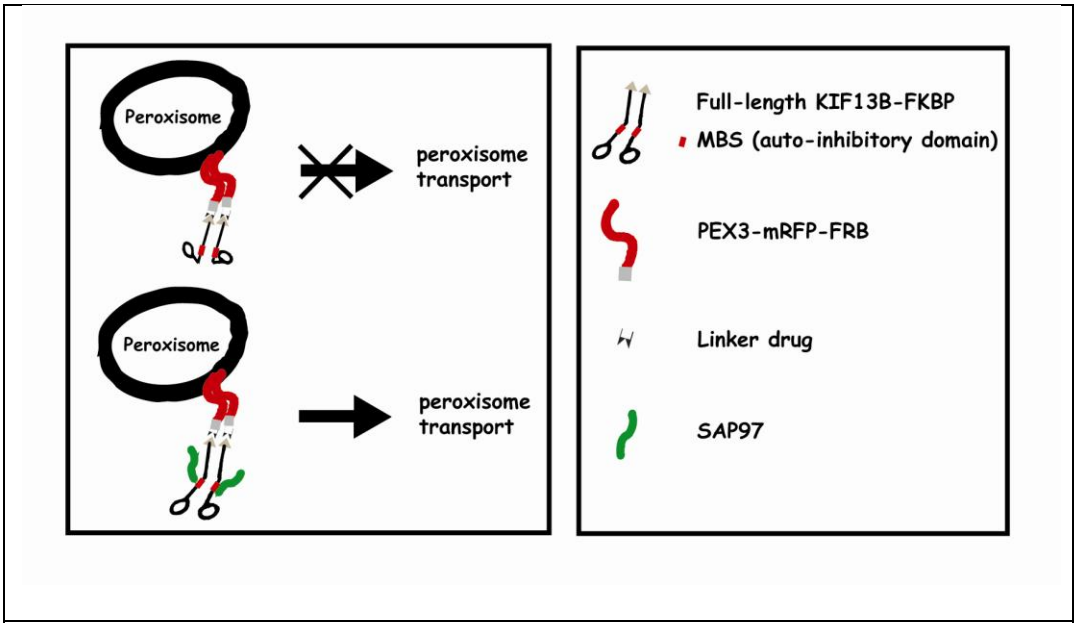


Figure 2: SAP97 as an activator of KIF13B in the peroxisome transport assay. Above: Co-expressing full-length KIF13B-FKBP and PEX3-mRFP-FRB results in no peroxisome transport. Below: Overexpression of SAP97 may activate KIF13B to allow peroxisome transport.

To test if SAP97 can bind to a similar vesicle population as KIF13B, I would create a SAP97-FRB and use the split-kinesin assay to test if SAP97 can bind to TfR vesicles. If it does, then TfR vesicles will enter the axon upon addition of the linker drug (Figure 3A). Results from experiments outlined in Figure 2 and Figure 3A would demonstrate that SAP97 can activate KIF13B and is also present on a KIF13B vesicle population. Taken together, these data would provide evidence to support the model that regulation of KIF13B activity may contribute to dendrite-selective transport.

Further experiments to determine whether KIF13B and SAP97 are on the same vesicle could be done as described above by co-expressing the GFP-13B tail, SAP97-FRB and motor-FKBP. If KIF13B tail and SAP97 are on the same vesicle, then after linking the motor and tail together, I should see GFP-13B labeled vesicles entering the axon (Figure 3B).

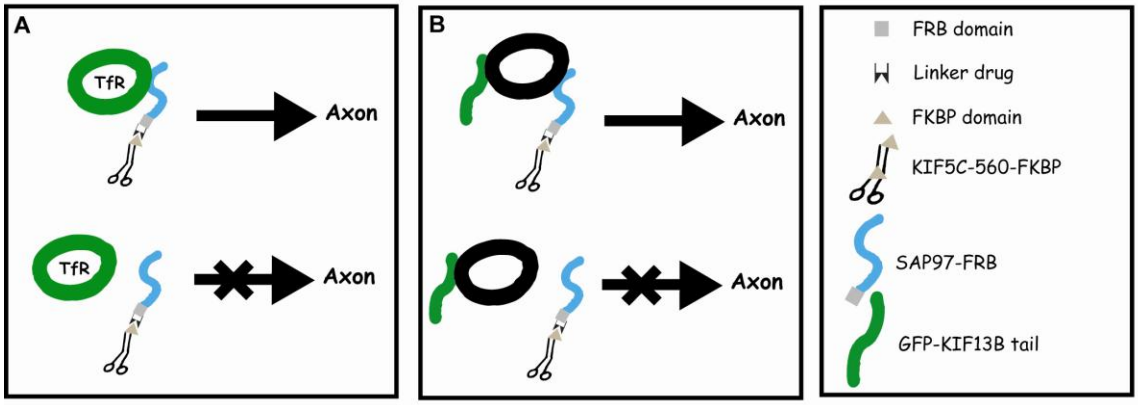


Figure 3: A, A SAP97-FRB will be used to determine if it can interact with vesicles labeled with TfR-GFP. B, A test to determine of KIF13B and SAP97 reside on the same vesicles. The unknown vesicle population will be labeled with GFP-KIF13B tail and would be transported into the axon after the addition of linker drug if SAP97 is present on the vesicle.

I have obtained preliminary data indicating that the regulation of KIF13B is involved in selective transport of TfR vesicles. I created a split-kinesin using the motor and tail domains of KIF13B. Unexpectedly, this split-kinesin moved TfR vesicles into the axon. This result may be explained if the intra-molecular interaction that regulates KIF13B inactivation is altered by introducing the FRB-FKBP domains. Similar to other split-kinesins, the KIF13B-KIF13B split kinesin is joined together directly after the motor domain. Since the motor domain is thought to bind the MBS region and this binding is thought to be necessary for regulation, the presence of the FRB-FKBP linkage may interfere with the intra-molecular interactions. To test this hypothesis, I propose to move this linker region to the C-terminal side of the MBS so the MBS can reach the motor domain for regulation.

However, for this hypothesis to be true, KIF13B needs to bind to its cargo beyond the MBS. Preliminary work from my colleagues, Marvin Bentley Ph.D. and Helena Decker Ph.D. in the Banker Lab has already narrowed the TfR-binding region to the C-terminal half of KIF13B, beyond the MBS. Since KIF13B binds to TfR vesicles in a region beyond the MBS, (leaving the autoinhibitory mechanism undisturbed) it is possible to test this idea. If this regulation is necessary, then I expect TfR vesicle to be transported by this KIF13B-KIF13B split-kinesin but not enter the axon as efficiently as previous split-kinesin combinations (Figure 4).

In summary, I have proposed several testable models to probe the mechanism of KIF13B-mediated dendrite-selective transport.

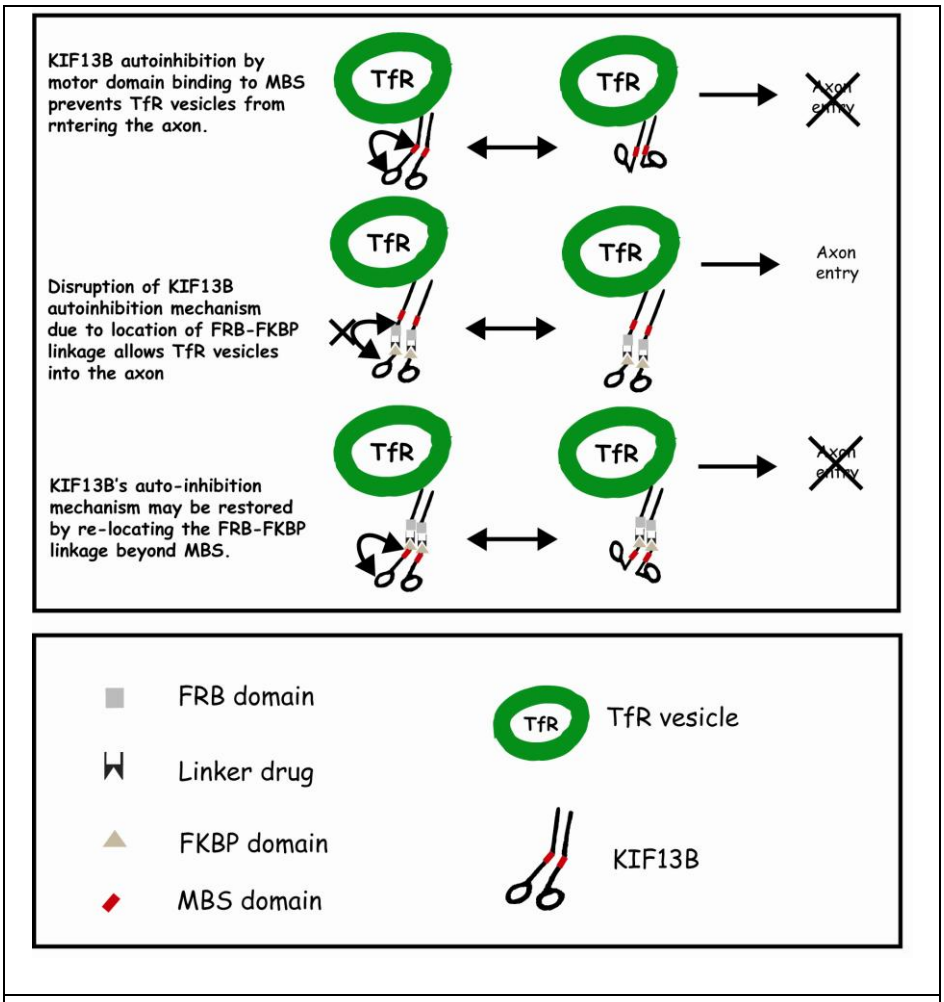


Figure 4: Intra-molecular interaction between the motor domain and the MBS contribute to dendrite-selective transport of KIF13B.

Axonal and dendritic microtubules and their cargo

In a final set of experiments related to the question of dendrite selective transport (but not related to KIF13B), I would investigate the potential roles of specific cargo as well as tubulin post-translational modifications (PTMs) in selective transport. It has been shown that KIF1A binds both a dendritic cargo (LDLR) and an axonal cargo (BDNF) (this work and [5]). How is it that a kinesin transports one cargo into the axon and another selectively in the dendrites? I hypothesize that KIF1A travels on different microtubule tracks, depending on whether it is attached to a dendritic or axonal vesicle. As a pilot experiment, I propose to observe transfected LDLR and BDNF (cargoes known to be transported by KIF1A) in fixed neurons stained for tubulin. Using super-resolution microscopy, I could then quantify how many vesicles of each type of vesicle population are associated with each microtubule. As individual microtubules may be difficult to visualize, I would search in regions of the neuron where microtubules are less bundled: soma, proximal dendrites and proximal axons. If dendritic and axonal vesicles are found to attach to the same stretch of microtubule in equal proportion, then this result would disprove my hypothesis. However, if I find that dendritic vesicles preferentially localize to microtubules not inhabited by axonal cargoes (and vice-versa), then I would proceed to investigate the possible differences between these sets of microtubules. For these studies, I plan to immunolabel for all known tubulin PTMs and dendritic microtubule-associated proteins and correlate each with dendritic vesicles. This result would be a first demonstration that axonal and dendritic vesicles associate with

separate and different populations of microtubules in a neuron. Next, I would proceed to immunolabel for endogenous KIF1A to show if it associates with axonal (BDNF) and dendritic (LDLR) vesicles on different microtubules. Verhey's group did similar experiments in fibroblasts and found kinesin-1 co-localized primarily with acetylated tubulin and that VSVG was transported preferentially on acetylated microtubules after leaving the Golgi [120]. These results suggest the feasibility of the proposed experiments and I would like to adapt these methods for use in neurons.

While the microtubule and vesicle population data may be correlative, it is an initial test to direct further investigations into whether microtubules contribute to selective transport. The mechanism for dendrite-selective transport may encompass contributions from each component of the kinesin-cargo-microtubule complex. The data presented and the experiments outlined in this thesis attempt to independently define the contributions of each to further our understanding of the mechanism for dendrite-selective transport.

References

1. Dotti, C.G., C.A. Sullivan, and G.A. Banker, *The establishment of polarity by hippocampal neurons in culture.* J Neurosci, 1988. **8**(4): p. 1454-68.
2. Bartlett, W.P. and G.A. Banker, *An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts.* J Neurosci, 1984. **4**(8): p. 1944-53.
3. Rasband, M.N., *The axon initial segment and the maintenance of neuronal polarity.* Nat Rev Neurosci. **11**(8): p. 552-62.
4. Noctor, S.C., et al., *Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases.* Nat Neurosci, 2004. **7**(2): p. 136-144.
5. Lo, K.Y., et al., *KIF1A is the primary anterograde motor protein required for the axonal transport of dense-core vesicles in cultured hippocampal neurons.* Neurosci Lett. **491**(3): p. 168-73.
6. Burack, M.A., M.A. Silverman, and G. Banker, *The role of selective transport in neuronal protein sorting.* Neuron, 2000. **26**(2): p. 465-72.
7. Cingolani, L.A. and Y. Goda, *Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy.* Nat Rev Neurosci, 2008. **9**(5): p. 344-356.
8. Anggono, V. and R.L. Huganir, *Regulation of AMPA receptor trafficking and synaptic plasticity.* Current Opinion in Neurobiology, (0).
9. Calabrese, B., M.S. Wilson, and S. Halpain, *Development and Regulation of Dendritic Spine Synapses.* Physiology, 2006. **21**(1): p. 38-47.
10. Ziv, N.E. and C.C. Garner, *Cellular and molecular mechanisms of presynaptic assembly.* Nat Rev Neurosci, 2004. **5**(5): p. 385-399.
11. Folsch, H., P.E. Mattila, and O.A. Weisz, *Taking the Scenic Route: Biosynthetic Traffic to the Plasma Membrane in Polarized Epithelial Cells.* Traffic, 2009.
12. Bonifacino, J.S. and B.S. Glick, *The Mechanisms of Vesicle Budding and Fusion.* Cell, 2004. **116**(2): p. 153-166.
13. Winckler, B., P. Forscher, and I. Mellman, *A diffusion barrier maintains distribution of membrane proteins in polarized neurons.* Nature, 1999. **397**(6721): p. 698-701.
14. Kobayashi, T., et al., *A functional barrier to movement of lipids in polarized neurons.* Nature, 1992. **359**(6396): p. 647-650.
15. Vale, R.D., *The molecular motor toolbox for intracellular transport.* Cell, 2003. **112**(4): p. 467-80.
16. Baas, P.W., et al., *Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite.* Proc Natl Acad Sci U S A, 1988. **85**(21): p. 8335-9.
17. Perlson, E., et al., *Retrograde axonal transport: pathways to cell death?* Trends in Neurosciences, 2010. **33**(7): p. 335-344.
18. Guillaud, L., M. Setou, and N. Hirokawa, *KIF17 dynamics and regulation of NR2B trafficking in hippocampal neurons.* J Neurosci, 2003. **23**(1): p. 131-40.
19. Setou, M., et al., *Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport.* Science, 2000. **288**(5472): p. 1796-802.
20. Black, M.M. and P.W. Baas, *The basis of polarity in neurons.* Trends Neurosci, 1989. **12**(6): p. 211-4.

21. Hirokawa, N., et al., *Kinesin superfamily motor proteins and intracellular transport.* Nat Rev Mol Cell Biol, 2009. **10**(10): p. 682-696.
22. Vale, R.D. and R.A. Milligan, *The Way Things Move: Looking Under the Hood of Molecular Motor Proteins.* Science, 2000. **288**(5463): p. 88-95.
23. Huang, C.F. and G. Banker, *The Translocation Selectivity of the Kinesins that Mediate Neuronal Organelle Transport.* Traffic.
24. Verhey, K.J. and J. Gaertig, *The tubulin code.* Cell Cycle, 2007. **6**(17): p. 2152-60.
25. Miki, H., Y. Okada, and N. Hirokawa, *Analysis of the kinesin superfamily: insights into structure and function.* Trends Cell Biol, 2005. **15**(9): p. 467-76.
26. Hirokawa, N. and R. Takemura, *Molecular motors and mechanisms of directional transport in neurons.* Nat Rev Neurosci, 2005. **6**(3): p. 201-14.
27. M.A. Silverman, S.K., X. Lu, M.R. Lasarev, S. Nagalla, and G. Banker, *Expression of Kinesin Superfamily Genes in Developing Hippocampal Neurons in Culture.* Manuscript in preparation, 2009.
28. Lawrence, C.J., et al., *A standardized kinesin nomenclature.* J Cell Biol, 2004. **167**(1): p. 19-22.
29. Bonifacino, J.S. and R. Rojas, *Retrograde transport from endosomes to the trans-Golgi network.* Nat Rev Mol Cell Biol, 2006. **7**(8): p. 568-579.
30. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-525.
31. Hirokawa, N. and Y. Noda, *Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics.* Physiol Rev, 2008. **88**(3): p. 1089-118.
32. Toyoshima, I., et al., *Kinectin, a major kinesin-binding protein on ER.* The Journal of Cell Biology, 1992. **118**(5): p. 1121-1131.
33. Vale, R.D., T.S. Reese, and M.P. Sheetz, *Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility.* Cell, 1985. **42**(1): p. 39-50.
34. Takeda, S., et al., *Kinesin Superfamily Protein 3 (Kif3) Motor Transports Fodrin-Associating Vesicles Important for Neurite Building.* The Journal of Cell Biology, 2000. **148**(6): p. 1255-1266.
35. Hanada, T., et al., *GAKIN, a novel kinesin-like protein associates with the human homologue of the Drosophila discs large tumor suppressor in T lymphocytes.* J Biol Chem, 2000. **275**(37): p. 28774-84.
36. Setou, M., et al., *Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites.* Nature, 2002. **417**(6884): p. 83-7.
37. Verhey, K.J., et al., *Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules.* J Cell Biol, 2001. **152**(5): p. 959-70.
38. Khodjakov, A., et al., *A Specific Light Chain of Kinesin Associates with Mitochondria in Cultured Cells.* Molecular Biology of the Cell, 1998. **9**(2): p. 333-343.
39. Glater, E.E., et al., *Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent.* The Journal of Cell Biology, 2006. **173**(4): p. 545-557.
40. Nakagawa, T., et al., *A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex.* Cell, 2000. **103**(4): p. 569-81.
41. Klopfenstein, D.R., et al., *Role of phosphatidylinositol(4,5)bisphosphate organization in membrane transport by the Unc104 kinesin motor.* Cell, 2002. **109**(3): p. 347-58.

42. Hoepfner, S., et al., *Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B.* Cell, 2005. **121**(3): p. 437-50.
43. Hall, D.H. and E.M. Hedgecock, *Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans.* Cell, 1991. **65**(5): p. 837-47.
44. Niwa, S., Y. Tanaka, and N. Hirokawa, *KIF1Bbeta- and KIF1A-mediated axonal transport of presynaptic regulator Rab3 occurs in a GTP-dependent manner through DENN/MADD.* Nat Cell Biol, 2008. **10**(11): p. 1269-79.
45. Donaldson, J.G. and C.L. Jackson, *ARF family G proteins and their regulators: roles in membrane transport, development and disease.* Nat Rev Mol Cell Biol. **12**(6): p. 362-375.
46. Allan, V.J., H.M. Thompson, and M.A. McNiven, *Motoring around the Golgi.* Nat Cell Biol, 2002. **4**(10): p. E236-E242.
47. Song, A.H., et al., *A selective filter for cytoplasmic transport at the axon initial segment.* Cell, 2009. **136**(6): p. 1148-60.
48. Chu, P.J., J.F. Rivera, and D.B. Arnold, *A role for Kif17 in transport of Kv4.2.* J Biol Chem, 2006. **281**(1): p. 365-73.
49. Shin, H., et al., *Association of the Kinesin Motor KIF1A with the Multimodular Protein Liprin-alpha.* Journal of Biological Chemistry, 2003. **278**(13): p. 11393-11401.
50. Marszalek, J.R., et al., *Novel dendritic kinesin sorting identified by different process targeting of two related kinesins: KIF21A and KIF21B.* J Cell Biol, 1999. **145**(3): p. 469-79.
51. Saito, N., et al., *KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles.* Neuron, 1997. **18**(3): p. 425-38.
52. Jareb, M. and G. Banker, *The polarized sorting of membrane proteins expressed in cultured hippocampal neurons using viral vectors.* Neuron, 1998. **20**(5): p. 855-67.
53. Reed, N.A., et al., *Microtubule acetylation promotes kinesin-1 binding and transport.* Curr Biol, 2006. **16**(21): p. 2166-72.
54. Jacobson, C., B. Schnapp, and G.A. Banker, *A change in the selective translocation of the Kinesin-1 motor domain marks the initial specification of the axon.* Neuron, 2006. **49**(6): p. 797-804.
55. Hammond, J.W., et al., *Posttranslational Modifications of Tubulin and the Polarized Transport of Kinesin-1 in Neurons.* Molecular Biology of the Cell. **21**(4): p. 572-583.
56. Sampo, B., et al., *Two distinct mechanisms target membrane proteins to the axonal surface.* Neuron, 2003. **37**(4): p. 611-24.
57. Kapitein, L.C., et al., *Mixed microtubules steer dynein-driven cargo transport into dendrites.* Curr Biol, 2010. **20**(4): p. 290-9.
58. Lewis, T.L., Jr., et al., *Myosin-dependent targeting of transmembrane proteins to neuronal dendrites.* Nat Neurosci, 2009. **12**(5): p. 568-76.
59. Kardon, J.R. and R.D. Vale, *Regulators of the cytoplasmic dynein motor.* Nat Rev Mol Cell Biol, 2009. **10**(12): p. 854-865.
60. Vallee, R.B., R.J. McKenney, and K.M. Ori-McKenney, *Multiple modes of cytoplasmic dynein regulation.* Nat Cell Biol. **14**(3): p. 224-230.
61. Wagner, W., S.D. Brenowitz, and J.A. Hammer, *Myosin-Va transports the endoplasmic reticulum into the dendritic spines of Purkinje neurons.* Nat Cell Biol, 2011. **13**(1): p. 40-48.
62. Eichler, T.W., et al., *The role of myosin Va in secretory granule trafficking and exocytosis.* Biochemical Society transactions, 2006. **34**(Pt 5): p. 671-4.

63. Nakagawa, T., et al., *Identification and classification of 16 new kinesin superfamily (KIF) proteins in mouse genome.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9654-9.
64. Okada, Y., et al., *The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors.* Cell, 1995. **81**(5): p. 769-80.
65. Tomishige, M., D.R. Klopfenstein, and R.D. Vale, *Conversion of Unc104/KIF1A Kinesin into a Processive Motor After Dimerization.* Science, 2002. **297**(5590): p. 2263-2267.
66. Rumbaugh, G., et al., *Synapse-associated protein-97 isoform-specific regulation of surface AMPA receptors and synaptic function in cultured neurons.* J Neurosci, 2003. **23**(11): p. 4567-76.
67. Schluter, O.M., W. Xu, and R.C. Malenka, *Alternative N-terminal domains of PSD-95 and SAP97 govern activity-dependent regulation of synaptic AMPA receptor function.* Neuron, 2006. **51**(1): p. 99-111.
68. Asaba, N., et al., *Direct interaction with a kinesin-related motor mediates transport of mammalian discs large tumor suppressor homologue in epithelial cells.* J Biol Chem, 2003. **278**(10): p. 8395-400.
69. Yamada, K.H., T. Hanada, and A.H. Chishti, *The effector domain of human Dlg tumor suppressor acts as a switch that relieves autoinhibition of kinesin-3 motor GAKIN/KIF13B.* Biochemistry, 2007. **46**(35): p. 10039-45.
70. Seiler, S., et al., *Cargo binding and regulatory sites in the tail of fungal conventional kinesin.* Nat Cell Biol, 2000. **2**(6): p. 333-8.
71. Hammond, J.W., et al., *Mammalian Kinesin-3 Motors Are Dimeric In Vivo and Move by Processive Motility upon Release of Autoinhibition.* PLoS Biol, 2009. **7**(3): p. e1000072.
72. Lee, J.R., et al., *An intramolecular interaction between the FHA domain and a coiled coil negatively regulates the kinesin motor KIF1A.* EMBO J, 2004. **23**(7): p. 1506-15.
73. Montgomery, J.M., P.L. Zamorano, and C.C. Garner, *MAGUKs in synapse assembly and function: an emerging view.* Cellular and Molecular Life Sciences, 2004. **61**(7): p. 911-929.
74. Thacker, E., et al., *The arf6 GAP centaurin alpha-1 is a neuronal actin-binding protein which also functions via GAP-independent activity to regulate the actin cytoskeleton.* Eur J Cell Biol, 2004. **83**(10): p. 541-54.
75. Moore, C.D., et al., *The neuronal Arf GAP centaurin alpha1 modulates dendritic differentiation.* J Cell Sci, 2007. **120**(Pt 15): p. 2683-93.
76. Miyazaki, H., et al., *The small GTPase ADP-ribosylation factor 6 negatively regulates dendritic spine formation.* FEBS Lett, 2005. **579**(30): p. 6834-8.
77. Venkateswarlu, K., T. Hanada, and A.H. Chishti, *Centaurin-alpha1 interacts directly with kinesin motor protein KIF13B.* J Cell Sci, 2005. **118**(Pt 11): p. 2471-84.
78. Durocher, D., et al., *The FHA Domain Is a Modular Phosphopeptide Recognition Motif.* Molecular Cell, 1999. **4**(3): p. 387-394.
79. Horiguchi, K., et al., *Transport of PIP3 by GAKIN, a kinesin-3 family protein, regulates neuronal cell polarity.* J Cell Biol, 2006. **174**(3): p. 425-36.
80. Tong, Y., et al., *Phosphorylation-independent dual-site binding of the FHA domain of KIF13 mediates phosphoinositide transport via centaurin Î±1.* Proceedings of the National Academy of Sciences, 2010. **107**(47): p. 20346-20351.

81. Huckaba, T.M., et al., *Kinesin-73 is a processive motor that localizes to Rab5-containing organelles.* J Biol Chem. **286**(9): p. 7457-67.
82. Yoshimura, Y., T. Terabayashi, and H. Miki, *Par1b/MARK2 phosphorylates kinesin-like motor protein GAKIN/KIF13B to regulate axon formation.* Mol Cell Biol. **30**(9): p. 2206-19.
83. Bolis, A., et al., *Dlg1, Sec8, and Mtmr2 regulate membrane homeostasis in schwann cell myelination.* J Neurosci, 2009. **29**(27): p. 8858-70.
84. Lamason, R.L., A. Kupfer, and J.L. Pomerantz, *The Dynamic Distribution of CARD11 at the Immunological Synapse Is Regulated by the Inhibitory Kinesin GAKIN.* Molecular Cell, 2010. **40**(5): p. 798-809.
85. Baas, P.W., M.M. Black, and G.A. Banker, *Changes in microtubule polarity orientation during the development of hippocampal neurons in culture.* J Cell Biol, 1989. **109**(6 Pt 1): p. 3085-94.
86. Silverman, M.A., et al., *Sorting and directed transport of membrane proteins during development of hippocampal neurons in culture.* Proceedings of the National Academy of Sciences, 2001. **98**(13): p. 7051-7057.
87. Lee, J.-R., et al., *Characterization of the Movement of the Kinesin Motor KIF1A in Living Cultured Neurons.* Journal of Biological Chemistry, 2003. **278**(4): p. 2624-2629.
88. Spencer, D., et al., *Controlling signal transduction with synthetic ligands.* Science, 1993. **262**(5136): p. 1019-1024.
89. Fujiki, Y., et al., *Import of peroxisomal membrane proteins: the interplay of Pex3p- and Pex19p-mediated interactions.* Biochim Biophys Acta, 2006. **1763**(12): p. 1639-46.
90. Platta, H.W. and R. Erdmann, *Peroxisomal dynamics.* Trends Cell Biol, 2007. **17**(10): p. 474-84.
91. Steinmetz, M.O. and A. Akhmanova, *Capturing protein tails by CAP-Gly domains.* Trends Biochem Sci, 2008. **33**(11): p. 535-45.
92. Peris, L., et al., *Tubulin tyrosination is a major factor affecting the recruitment of CAP-Gly proteins at microtubule plus ends.* J Cell Biol, 2006. **174**(6): p. 839-49.
93. Al-Bassam, J., et al., *Distinct conformations of the kinesin Unc104 neck regulate a monomer to dimer motor transition.* The Journal of Cell Biology, 2003. **163**(4): p. 743-753.
94. Kaech, S. and G. Banker, *Culturing hippocampal neurons.* Nat Protoc, 2006. **1**(5): p. 2406-15.
95. Hirokawa, N., S. Niwa, and Y. Tanaka, *Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease.* Neuron, 2010. **68**(4): p. 610-38.
96. Silverman, M.A., et al., *Sorting and directed transport of membrane proteins during development of hippocampal neurons in culture.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7051-7.
97. Nakata, T. and N. Hirokawa, *Microtubules provide directional cues for polarized axonal transport through interaction with kinesin motor head.* J Cell Biol, 2003. **162**(6): p. 1045-55.
98. Henthorn, K.S., et al., *A Role for Kinesin Heavy Chain in Controlling Vesicle Transport into Dendrites in Drosophila.* Mol Biol Cell, 2011.
99. Goldstein, L.S. and Z. Yang, *Microtubule-based transport systems in neurons: the roles of kinesins and dyneins.* Annu Rev Neurosci, 2000. **23**: p. 39-71.
100. Silverman, M.A., et al., *Expression of kinesin superfamily genes in cultured hippocampal neurons.* Cytoskeleton (Hoboken), 2010. **67**(12): p. 784-95.

101. Huang, C.F. and G. Banker, *The Translocation Selectivity of Kinesins that Mediate Organelle Transport in Culture Neurons.* Under Review, 2011.
102. Nakata, T., et al., *Preferential binding of a kinesin-1 motor to GTP-tubulin-rich microtubules underlies polarized vesicle transport.* J Cell Biol, 2011.
103. Lee, J.R., et al., *Characterization of the movement of the kinesin motor KIF1A in living cultured neurons.* J Biol Chem, 2003. **278**(4): p. 2624-9.
104. Lochner, J.E., et al., *Real-time imaging of the axonal transport of granules containing a tissue plasminogen activator/green fluorescent protein hybrid.* Mol Biol Cell, 1998. **9**(9): p. 2463-76.
105. Lo, K.Y., et al., *KIF1A is the primary anterograde motor protein required for the axonal transport of dense-core vesicles in cultured hippocampal neurons.* Neurosci Lett, 2011. **491**(3): p. 168-73.
106. Rivera, J., et al., *The role of Kif5B in axonal localization of Kv1 K(+) channels.* Eur J Neurosci, 2007. **25**(1): p. 136-46.
107. Kennedy, M.J., et al., *Syntaxin-4 defines a domain for activity-dependent exocytosis in dendritic spines.* Cell. **141**(3): p. 524-35.
108. May, P., et al., *The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions.* Ann Med, 2007. **39**(3): p. 219-28.
109. Gan, Y., T.E. McGraw, and E. Rodriguez-Boulan, *The epithelial-specific adaptor AP1B mediates post-endocytic recycling to the basolateral membrane.* Nat Cell Biol, 2002. **4**(8): p. 605-9.
110. Maxfield, F.R. and T.E. McGraw, *Endocytic recycling.* Nat Rev Mol Cell Biol, 2004. **5**(2): p. 121-32.
111. Akhmanova, A. and J.A. Hammer, 3rd, *Linking molecular motors to membrane cargo.* Curr Opin Cell Biol, 2010. **22**(4): p. 479-87.
112. Yonekawa, Y., et al., *Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice.* J Cell Biol, 1998. **141**(2): p. 431-41.
113. Yoshimura, Y., T. Terabayashi, and H. Miki, *Par1b/MARK2 phosphorylates kinesin-like motor protein GAKIN/KIF13B to regulate axon formation.* Mol Cell Biol, 2010. **30**(9): p. 2206-19.
114. Leterrier, C., et al., *End-binding proteins EB3 and EB1 link microtubules to ankyrin G in the axon initial segment.* Proc Natl Acad Sci U S A, 2010. **108**(21): p. 8826-31.
115. Muthuswamy, S.K., M. Gilman, and J.S. Brugge, *Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers.* Mol Cell Biol, 1999. **19**(10): p. 6845-57.
116. Chua, J.J., et al., *Phosphorylation-regulated axonal dependent transport of syntaxin 1 is mediated by a Kinesin-1 adapter.* Proc Natl Acad Sci U S A, 2012. **109**(15): p. 5862-7.
117. Morfini, G., et al., *Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility.* EMBO J, 2002. **21**(3): p. 281-293.
118. Toda, H., et al., *UNC-51/ATG1 kinase regulates axonal transport by mediating motor-cargo assembly.* Genes Dev, 2008. **22**(23): p. 3292-307.
119. Wang, X. and T.L. Schwarz, *The Mechanism of Ca2+-Dependent Regulation of Kinesin-Mediated Mitochondrial Motility.* Cell, 2009. **136**(1): p. 163-174.
120. Cai, D., et al., *Single Molecule Imaging Reveals Differences in Microtubule Track Selection Between Kinesin Motors.* PLoS Biol, 2009. **7**(10): p. e1000216.