AXONAL TRANSPORT OF NEUROPEPTIDES IN *DROSOPHILA* AXONS

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by

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Table of Contents

List of Figures and Tables v

ABSTRACT vii

Dedication and Acknowledgement ix

Chapter 1: Introduction 1

1.1 Axonal Transport 1

1.2 Cytoskeletal Microtubules are “tracks” for fast axonal transport 5

1.3 Motor Proteins drive Fast Axonal Transport 10

1.3.1 The Dynein Family 13

1.3.2 The Kinesin Superfamily 18

1.3.3 Cellular Functions of the Kinesin Superfamily 19

1.3.4 Kinesin-1 20

1.4 Current Questions about the mechanisms of axonal transport 26

1.5 Dense Core Vesicles 27

Chapter 2: Motor Proteins in Axonal Transport of Neuropeptide Vesicles 31

2.1 Introduction 31

2.2 Materials and Methods 35

2.3.1 Kinesin-1 is required for DCV distribution and transport in neurons 40

2.3.2 Evidence for a direct Kinesin-1 DCV relationship 48

2.3.3 Dynein is required for DCV transport in both directions 52

2.4 Discussion 58

2.4.1 Opposing MT motor interdependence: 59
2.4.2 A requirement for two DCV kinesins

Chapter 3 Autophagy and Axonal Swellings

3.1 Introduction

3.2 Materials and Methods

3.3 Results

3.3.1 Autophagic membranes are localized in focal accumulations in axons

3.3.2 TOR inhibition reduces axonal swellings

3.3.3 TOR activation increases axonal swellings

3.3.4 Atg1 overexpression suppresses axonal swellings

3.4 Discussion and Future Experiments

Supplemental Files

Movie 1
Movie 2
Movie 3
Movie 4
Movie 5
Movie 6
Movie 7
Movie 8

References
**LIST OF FIGURES AND TABLES**

**FIGURE 1-1** A composite diagram showing fiber deformations proximal to a constriction. .......................................................... 4

**FIGURE 1-2** Electron micrographs showing examples of vesicles in close proximity to a microtubule filament. .......................................................... 6

**FIGURE 1-3** Electron micrograph of a cat lumbar colonic nerve section viewed with the distal (synaptic terminal) side away from the viewer. 9

**FIGURE 1-4** Figure showing the general structures of the 3 microtubule motor family types.......................................................... 12

**FIGURE 1-5** A 3-D model of a single cytoplasmic dynein heavy chain bound to a microtubule.......................................................... 15

**FIGURE 1-6** Generation of a power stroke by the Dhc linker domain (purple). .................................................................................. 16

**FIGURE 1-7**. Figure showing the general structure of kinesin-1. ............... 22

**FIGURE 1-9** Two distinct secretory pathways are present in neuroendocrine and endocrine cells. .......................................................... 30

**FIGURE 2-1** Kinesin-1 influences the distribution and flux of axonal DCVs. 44

**FIGURE 2-2** Kinesin-1 influences anterograde DCV run behaviors. .......... 46

**FIGURE 2-3** Milton does not influence anterograde DCV run behavior...... 50

**FIGURE 2-4** Co-fractionation of kinesin-1 and kinesin-3 with DCVs........... 51

**FIGURE 2-5** Influence of Dynein on the distribution and flux of DCVs....... 53

**FIGURE 2-6** Dynein influences axonal DCV run behaviors. .................... 56

**FIGURE 3-1** Mutations in kinesin-1 and dynein cause swellings in axons. ... 67

**FIGURE 3-2** Macropathology in mammalian cells........................................ 70

**FIGURE 3-3** TOR signaling pathway in mammals. ................................... 71

**FIGURE 3-4** mCherry-Atg8 is detected in axonal swellings. ..................... 76
FIGURE 3-5 DESIGN OF RNAi SCREEN TO IDENTIFY MODIFIERS OF AXONAL SWELLINGS. ................................................................. 77

FIGURE 3-6 A DECREASE IN AXONAL SWELLINGS IN LIVING TOR RNAi LARVAE. .... 79

FIGURE 3-7 TOR RNAi EFFECTS SEEN IN DISSECTED AND FIXED LARVAL NEUROMUSCULAR SYSTEMS. ............................................................................. 80

FIGURE 3-8 RHEB OVEREXPRESSION INCREASES THE NUMBER AND SIZE OF AXONAL SWELLINGS IN LARVAE OVER EXPRESSING APLIP1. .......................................................... 83

FIGURE 3-9 ATG1 OVEREXPRESSION DECREASES THE SIZE AND NUMBER OF AXONAL SWELLINGS IN LARVAE OVEREXPRESSING APLIP1. .................................................... 84

TABLE 1 KINESIN SUPERFAMILY PROTEINS AND SOME OF THEIR REPORTED FUNCTIONS. .................................................................................. 25

TABLE 2 DCV TRANSPORT PARAMETERS IN DROSOPHILA AXONS. .................. 43
ABSTRACT

Angeline Lim

Transport of Neuropeptides in Drosophila Axons

Microtubule motor proteins are known to drive long distance organelle transport in neurons, but there are many motor species and many organelle types, so specific transport mechanisms remain largely undefined. To gain insight into the transport mechanism for dense core vesicles (DCV) that carry neuropeptides, we studied three motors in axons. Our prior work showed that inhibition of Unc-104 (kinesin-3) greatly reduced anterograde and retrograde DCV flux in motor axons, and caused defects in both anterograde and retrograde run parameters (duty cycles, run velocities, and run lengths). Here, using time-lapse imaging of whole, live Drosophila larvae, we report that inhibition of Khc (kinesin-1), well known as a mitochondrial motor, reduces DCV flux in both directions, but inhibits just anterograde run parameters. Specific inhibition of Khc-driven mitochondrial transport by Milton RNAi had little influence on DCV transport, and sedimentation tests showed that both kinesin-1 and -3 co-fractionate with DCVs. These findings suggest that both kinesin-1 and -3 contribute directly to DCV transport, that their functions on anterograde DCVs are interdependent, and that their influences on the retrograde DCV motor are distinct. Additional tests identified cytoplasmic dynein as a third motor and showed that it is needed for anterograde as well as retrograde
transport. Overall, our data suggest a mechanism in which both kinesins have interdependent but distinct roles in DCV distribution -- kinesin-3 has a major role in moving neuropeptide DCVs from the cell body into axons. Then kinesin-1 and -3 work together in a dynein-dependent manner to drive highly processive DCV transport toward the axon terminal. Dynein, activated by one or both kinesins, carries excess DCVs that have not been secreted back to the cell body.
DEDICATION AND ACKNOWLEDGEMENT

This dissertation is dedicated to my mother, whose strength and perseverance allowed me to be where I am today. And to my son, Anders, for all the love and laughter that keeps me going.

I would not have been able to complete this thesis without the help and encouragement of family and friends. In particular, I would like to thank my advisor, Prof. William Saxton for his guidance throughout these years. He is an excellent mentor and taught me about scientific rigor, integrity, and attention to details while keeping the big picture in perspective. “What is the question?” he would say. Although Bill has a more “hands-off” mentoring style, he has an uncanny ability to know when his students need a boost of encouragement. There were times when I felt that my project was going nowhere and Bill would, at the appropriate moment, start throwing ideas around or just say something positive. Bill’s dedication to doing good science, and being a good, nurturing mentor are qualities that I hope to have someday.

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CHAPTER 1 : INTRODUCTION

1.1 AXONAL TRANSPORT

Neurons are undeniably one of the most morphologically and functionally asymmetrical cell types. A typical neuron has a nucleus-containing cell body from which multiple processes project (neurites). Among these neurites, there is usually a single long axon that branches into presynaptic terminals that include rounded structures known as boutons. Dendrites, the other non-axonal neurites, tend to be shorter with extensive branched structures. Dendrites receive information from other neurons or sensory cells and the axon integrates and propagates the information as action potentials over long distances rapidly. The action potential reaches the synaptic bouton and causes the release of chemical neurotransmitters or small polypeptides into the synapse, which then transmit the signals to dendrites of neighboring neurons.

Because the synaptic terminal and its boutons tend to be located far from the cell body, neurons are heavily dependent on axonal transport to provide the terminal with an uninterrupted supply of materials synthesized in the cell body. This ensures a steady stream of organelles and neurotrophic factors that maintains the synapse and allows for neurotransmission. Similarly, axonal transport allows excess materials,
senescent organelles and components of the endocytic pathway to make their way from the terminal and axon to the cell body for signaling purposes, recycling or degradation (reviewed in (Franker and Hoogenraad, 2013)).

The first experimental evidence of axonal transport came from a seminal work by Paul Weiss and Helen Hiscoe in 1948 (Weiss and Hiscoe, 1948). In their studies, when a partial constriction was placed around a nerve, the axons proximal to the constriction swelled dramatically. This led them to postulate that there was a flow of material from the cell body that, when obstructed by a constriction, swelled like water behind a dam (Figure 1-1). Components of the “dammed up” materials were later identified as membrane bound vesicles such as dense core vesicles and (Van Breemen et al., 1958) enzymes such as acetylcholinesterase, acetyltransferase (Friede, 1959) and proteins (Koenig, 1958; Droz and Leblond, 1962; Koenig and Droz, 1971). Swellings were also observed in axons distal to constriction sites, suggesting that axonal transport is bidirectional (Lasek, 1967; Zelena, 1968; Lubinska, 1971). This was further confirmed in isolated axons that showed organelles moving in both directions (Cooper and Smith, 1974). Thus, axonal transport functions as the “supply line” between the neuronal cell body with its nucleus and the distal parts of the axon.

Following the discovery by Weiss and Hiscoe in 1948, many key advances have contributed to identifying the components required for axonal transport. These studies include the identification of microtubules as the cytoskeletal structure
essential for long-distance, fast axonal transport and the discovery of families of force-generating motor proteins that bind to and transport various cargoes such as mitochondria, vesicles, degradative organelles, and mRNA particles (mRNP). The following sections will provide an overview of axonal microtubules, motor proteins and on a specific type of transported cargo, neuropeptide-carrying dense core vesicles (DCVs).
Figure 1-1 A composite diagram showing fiber deformations proximal to a constriction. The black box represents the site of constriction. “Deformations” of axons (grey) were seen proximal on the cell body side of the constriction (left). This figure was adapted from Weiss and Hiscoe (Weiss and Hiscoe, 1948).
1.2 CYTOSKELETAL MICROTUBULES ARE “TRACKS” FOR FAST AXONAL TRANSPORT

The cytoplasm of a cell is organized by the cytoskeleton, a dynamic matrix of polymers that is critical for maintaining cell shape, division and transport. The neuronal cytoskeleton is comprised of microtubules, microfilaments and neurofilaments. Microfilaments are polymers of actin that form a mesh like, branched structure. They are abundant beneath the axonal plasma membrane, in the axon hillock, in growth cones and in nerve terminals (Goldman, 1983; Matus et al., 1982; Watanabe et al., 2013). In contrast, microtubules and neurofilaments form long, unbranched polymers aligned parallel to the length of the axon (reviewed in (Brown, 2013)). As early as 1971, drawing on an analogy from the acto-myosin system in muscle contraction, it was proposed that organelles bind to filaments with mobile cross bridges for fast axonal transport (Ochs, 1971, 1974). Biochemical and microscopy studies supported a role for microtubules as “tracks” for axonal transport. A key finding was that drugs or treatments that disrupted microtubules also inhibited fast transport (Banks et al., 1971; Edstrom and Hanson, 1975; Friede and Ho, 1977; Hammond and Smith, 1977). In addition, electron microscope ultrastructural analyses of axons revealed frequent association of moving organelles with microtubules, but not with neurofilaments (Figure 1-2) (Miller and Lasek, 1985)
Figure 1-2 Electron micrographs showing examples of vesicles in close proximity to a microtubule filament. The arrowhead points to a cross bridge between a vesicle and a microtubule. Small arrows point to structures similar to cross bridges extending from vesicles, but not attached to microtubules. Scale bar = 0.25um. Figure adapted from (Miller and Lasek, 1985).
One of the unique features of the neuron lies in the organization of microtubules in the axon. Polymers are formed from a head-to-tail association of $\alpha/\beta$-tubulin dimers that gives rise to microtubules with a structural polarity. The microtubule end with $\beta$-tubulin exposed is known as the “plus” end while $\alpha$-tubulin marks the “minus” end. The original technique for determining microtubule orientation, developed by Heidemann and McIntosh, is known as hooking (Heidemann and McIntosh, 1980). Under specific buffer conditions, tubulin subunits preferentially add all along the sides of microtubules instead of at the ends. The new protofilaments curve around the existing microtubule and, when viewed in cross section, curve like hooks decorating the microtubule. When viewed with electron microscopy, microtubule sections with clockwise hooks correspond to the plus end pointing away from the observer (Figure 1-3). In axons, microtubules have their plus ends oriented away from the cell body, pointing toward the distal terminals (Burton and Paige, 1981; Heidemann et al., 1981). This uniform polarity is important for determining the directionality for axonal transport, as will be discussed later. It is also essential to note that although microtubules can be very long, they are not continuous along the entire length of the axon. Instead, there is considerable overlap of shorter microtubule filaments that ensures uninterrupted transport.

In contrast, the orientation of microtubules in dendrites is less uniform than in axons. Olfactory neurons in frogs (Burton, 1985) (Burton and Paige, 1981) and retinal cone cells in teleosts (Troutt and Burnside, 1988) have dendrites with microtubule
minus-ends away from the cell body. However, later studies in cultured rat hippocampal neurons and sympathetic neurons showed dendrites with microtubules of mixed polarity. The distal dendritic tips have mostly plus-end out microtubules while dendrites proximal to the cell body have mostly minus-end out microtubules (Wang et al., 1996). However in *Drosophila* neurons, almost all microtubules in dendrites are oriented minus-ends out, while axons contain microtubules with their plus-ends out (Stone et al., 2008). Thus it appears that the orientation of microtubules in axons (plus-end out/ distal) is conserved in both vertebrate and invertebrate neurons but the same cannot be said for dendrites. The uniform orientation of axonal microtubules, coupled with the extreme lengths and narrow widths of axons, provides an ideal situation for the study of intracellular transport.
Figure 1-3 Electron micrograph of a cat lumbar colonic nerve section viewed with the distal (synaptic terminal) side away from the viewer.
Tubulin “hooks” show microtubule orientation. The clockwise hooks (arrow) show microtubules with plus ends pointed away from the viewer. In these axons, ~68% of the microtubules display hooks, with >95% of those showing clockwise hooks. An arrowhead points to an example of a rare counterclockwise hook. Image adapted from (Heidemann et al., 1981).
1.3 MOTOR PROTEINS DRIVE FAST AXONAL TRANSPORT

Motor proteins are the force generating molecules responsible for intracellular transport. There are three main classes of motor proteins – kinesins and dyneins, which bind microtubules, and myosins, which bind microfilaments (reviewed in (Woehlke and Schliwa, 2000)). All motor proteins studied to date are unidirectional. Most kinesins move toward the plus-ends and dyneins move toward the minus ends of microtubules (reviewed in (Woehlke and Schliwa, 2000)). Most myosins move toward plus-ends of microfilaments, except for Myosin VI, which is minus-end directed.

While there are many differences between these three motors, they also share some general features that make them ideal for transport functions. Each motor is a multimeric protein complex consisting of two force-generating heavy chains that usually bind associated proteins that serve as cargo likers and/or regulators. The heavy chain includes a globular ATPase motor domain that creates force and motion along with a stalk/tail region that confers functional diversity (Figure 1-4). The globular head is small for kinesins (~350 amino acids), larger for myosin (~800 amino acids) and largest for dyneins (~4000 amino acids). ATP hydrolysis in a head results in a conformation change that shifts the stalk-tail forward along a microtubule or microfilament. The dimerization of heavy chains allows them to move processively (continuously) for relatively long distances along microtubules. For
example, kinesin-1 moves in a “hand-over-hand” manner using its 2 heavy chains. When a motor domain binds a microtubule, ADP is released, ATP binds, and the partner motor domain shifts forward (toward the plus end) to find the next available binding site on the microtubule (Yildiz et al., 2004).

The organization of filaments is a crucial element of all motility processes. Because microfilaments are in a random meshwork and microtubules are arranged in long unipolar bundles in axons, it is reasonable to suspect that the long distance axonal transport motors are kinesins and dyneins. Indeed, many studies have shown that inhibition of kinesins or dyneins have dramatic effects in fast axonal transport (Brady et al., 1990; Waterman-Storer et al., 1997; Martin et al., 1999; Pilling et al., 2006; Barkus et al., 2008; Djagaeva et al., 2013). There is little evidence supporting a direct role of myosin in fast axonal transport. Instead, studies have suggested that myosin can control the interaction of kinesin/dynein cargos with microtubules and that it is important for short range movement and anchoring of cargos in actin rich areas near the synaptic terminals (Ali et al., 2008). My thesis work has focused on long-distance fast axonal transport driven by kinesin and dynein, so the following sections will focus on them.
Figure 1-4 Figure showing the general structures of the 3 microtubule motor family types. All 3 motors consist of a heavy chain dimer. The catalytic domain (yellow) binds and hydrolyzes ATP. The stalk (blue) forms extended coiled-coils in myosin and kinesin. Each motor usually has associated polypeptides (pink) that are important for cargo recognition. Figure adapted from a review by (Woehlke and Schliwa, 2000).
1.3.1 The Dynein Family

Dynein was originally identified as an ATPase in the cilia of *Tetrahymena pyriformis* and was named from “dyne” – a common unit of force (Gibbons, 1963; Gibbons and Rowe, 1965). The isolation of other dynein-like proteins in the cytoplasm suggested a dynein family of proteins that were either associated with axonemes in cilia and flagellar, or associated with non-axonemal microtubules in cytoplasm (Weisenberg and Taylor, 1968; Pratt, 1980). Later, cytoplasmic ATPases from bovine brain and from *C. elegans* were characterized and found to be similar in size and biochemistry to axonemal dynein (Lye et al., 1987; Paschal et al., 1987). The bovine brain ATPase, MAP1C, which is now known as cytoplasmic dynein was subsequently shown to be a minus-end directed motor thought to be responsible for retrograde fast axonal transport (Paschal and Vallee, 1987).

Dynein belongs to the AAA+ superfamily (ATPase Associated with diverse Activities). Dynein heavy chain (Dhc), the force producing subunit is a massive protein of ~500kDa. Two Dhcs dimerize through their amino-terminal tails that also provide a platform for binding to several other associated subunits that mediate cargo interactions either directly or by the recruitment of other adaptor proteins. The motor domain of Dhc is a hexameric ring of 6 AAA+ modules (AAA1 to AAA6). AAA1 – AAA4 have nucleotide binding capability. Mutagenesis and biochemical experiments suggest that AAA1 contains the force-generating ATP binding pocket. An anti-
parallel coiled-coil stalk protruding from AAA4 binds microtubules in an ATP-sensitive manner. Another protrusion from the adjacent AAA5, called the strut (buttress) interacts with the stalk (Figure 1-5). Through the AAA+ ring, ATP hydrolysis in AAA1 produces long range structural changes resulting in a power stroke (Roberts et al., 2013). The main mechanical element behind the power stroke is the linker domain arching over the AAA+ ring. Based on various structures from *D. discoideum, S. cerevisiae* and *C. reinhardtii*, a model for how the linker generates the power stroke has been proposed (Figure 1-6). In an ATP bound state, the linker arches over and interacts with AAA2. The power stroke is initiated by AAA1 ATP hydrolysis, which causes the linker to straighten and lie over AAA4, before docking at AAA5. Completion of the power stroke occurs with the loss of ADP (Kon et al., 2012; Roberts et al., 2012; Schmidt et al., 2012; Roberts et al., 2013).
Figure 1-5 A 3-D model of a single cytoplasmic dynein heavy chain bound to a microtubule. Because there is not yet a high resolution structure for Dhc bound to microtubules, this model is a composite of the crystal structure of D. discoideum Dhc and a cryo-EM model of the mouse Dhc microtubule-binding domain bound to tubulin. The 6 AAA+ modules are numbered (1-6). Model adapted from (Roberts et al., 2013).
Figure 1-6 Generation of a power stroke by the Dhc linker domain (purple). During the ATP bound state, the linker is bent towards AAA2 and is mobile (purple arcs). The red lines represent 2 hairpins in AAA2 that contact the linker (left). Release of the ATP gamma phosphate drives structural changes, resulting in the power stroke. The linker now lies over AAA4 (middle image, yellow). When the linker docks at AAA5 (right), the ATP binding pocket in AAA1 (dark blue) becomes “open” allowing ADP to leave. See text for details. Figure adapted from (Roberts et al., 2013)
Phylogenetic analysis has identified 9 major classes of dynein (Wickstead and Gull, 2007). Two of them are cytoplasmic dyneins (DYNC1H1 and DYNC2H1 in humans). DYNC1H1 is used in nearly all minus-end directed transport in eukaryotic cell cytoplasm. DYNC2H1 is found in cells that build axonemes and is a motor for intraflagellar transport. The remaining 7 major dynein classes are axonemal dyneins required for ciliary beating. I studied DYNC1H1 in my thesis research, and will refer to it simply as dynein or Dhc.

The function diversity of dynein has been attributed mainly to the huge array of subunits that associate with the dynein tail complex. There are 5 known classes of subunits, the intermediate chain, the light intermediate chain, and 3 light chains (TCTEX, LC8 and Roadblock). In addition, there are three regulators known to associate with dynein, LIS1, NUDE and the dynactin complex (Roberts et al., 2013).

The main cellular functions of dynein can be assigned to three categories, namely, organelle transport, organelle positioning and cell division. Being the main minus-end directed microtubule motor in most cells, dynein is involved in moving a number of organelles such as mitochondria (Pilling et al., 2006), endosomes (Driskell et al., 2007), lysosomes (Jordens et al., 2001), melanosomes (Gross et al., 2002), peroxisomes (Kural et al., 2005), lipid droplets (Gross et al., 2000) and autophagosomes (Kimura et al., 2008). It is also involved in ER to Golgi trafficking (Presley et al., 1997) and endosome fusion (Aniento et al., 1993). Dynein is
important for the maintenance of organelle structure. Disruption of the dynein/dynactin interaction results in dispersion of the Golgi complex (Burkhardt et al., 1997). Dynein plays a number of essential roles in cell division. It is required for spindle assembly and chromosome segregation; it localizes to the kinetochore and is thought to have a checkpoint function by delaying anaphase until all chromosomes are attached to the spindle (Howell et al., 2001).

1.3.2 The Kinesin Superfamily

The first kinesin (Kinesin-1/Khc/conventional kinesin) was isolated from squid axoplasm and bovine brains (Brady, 1985; Vale et al., 1985). Subsequently, kinesin has been isolated from other organisms, including sea urchin (Scholey et al., 1985), Drosophila (Saxton et al., 1988) and pig (Amos, 1987). Some of the initial hints of an extended kinesin family came from analysis of proteins involved in cell division (Enos and Morris, 1990) and nuclear fusion (Meluh and Rose, 1990). Genomic approaches have since identified kinesin homologs in all eukaryotes (Wickstead and Gull, 2006). All kinesins contain a 350 amino acid region that is homologous to the catalytic or "motor" domain of Drosophila kinesin-1. Beyond the motor domain, kinesin related proteins show no similarity to kinesin-1 suggesting that the highly conserved motor domain has been adapted for many functions. The number of kinesin families has expanded to 14-16 plus 1 orphan class (Lawrence 2004; Wickstead, 2006). Members of each kinesin family have similar domain
organization, share some common sequences outside the motor domain, move with comparable rates and have similar cellular functions.

1.3.3 Cellular Functions of the Kinesin Superfamily

Kinesins generate force for many cellular processes, including organelle transport, intraflagellar transport, chromosome alignment and spindle formation (Table 1) (reviewed by (Endow, 1993; Hirokawa et al., 1998)). Because axonal transport is the focus of this thesis, the following sections will focus on the organelle kinesins. Three kinesin families, kinesin-1, -2 and -3, are clearly involved in cytoplasmic organelle transport (Moore and Endow, 1996). Kinesin-1 was originally discovered as an anterograde motor in neurons (Brady, 1985; Vale et al., 1985). Kinesin-2 motors function in cilia where they drive anterograde intraflagellar transport (Scholey, 2013) but they also contribute to cytoplasmic organelle transport: e.g. the movement of melanophores in *Xenopus* (Rogers et al., 1997) and the transport of choline acetyltransferase in *Drosophila* axons (Ray et al., 1999). Kinesin-3 is involved in transport of vesicles that carry neuropeptides and membrane proteins to the synaptic terminals of axons (Hall et al., 1993; Barkus et al., 2008). There have been a few reports suggesting that other members of the kinesin family have roles in cytoplasmic organelle transport but their functions seem generally restricted to only 1 species. Kif4, a kinesin-4 in mouse is concentrated in growth cones and may be involved in axonal transport (Sekine et al., 1994). Kinesin-8 (klp67A) was found co-
localized with some mitochondria in dividing cells, but its primary function is not in long distance mitochondria transport in post-mitotic axons (Pereira et al., 1997). My thesis work touches on kinesins -2 and -3, but has focused more on kinesin-1.

1.3.4 Kinesin-1

In the early 1980s, the molecular mechanism of fast axonal transport was unknown. At that time, the only known force producing ATPases were myosin-II (actomyosin muscle contraction motor) and axonemal dynein (flagella and cilia bending motor). A key finding that pointed to the presence of a yet unidentified translocator came from studies showing that AMP-PNP, a non-hydrolyzable analogue of ATP, stopped vesicle transport in axoplasm leaving vesicles bound to microtubules (Lasek and Brady, 1985). In contrast, AMP-PNP caused dissociation of myosin and axonemal dynein from actomyosin and microtubules respectively (Greene and Eisenberg, 1980; Satir et al., 1981). The unknown axoplasmic ATPase, with enzymatic properties different from myosin or dynein was subsequently identified by 2 different groups as a microtubule-stimulated ATPase, and named Kinesin (now called kinesin-1) (Brady, 1985; Vale et al., 1985).

Structure: Kinesin-1 is a heterotetramer made up of 2 identical heavy chains (~110kd) and 2 light chains (~70kd). The kinesin heavy chain has 3 distinct regions: a highly conserved motor domain at the amino terminal, an alpha-helical stalk, and a
globular tail at the carboxyl terminal (Figure 1-7). The ~350 amino acid motor domain is the main force generating site, containing the ATPase and microtubule binding activities (Yang et al., 1990). Single molecule studies using optical traps have shown that dimerized kinesin heads walk along microtubules with an 8nm step size, which corresponds to the size of a tubulin dimer (Svoboda et al., 1993; Yildiz and Selvin, 2005). Dimerization of Khc is mediated by the stalk, which is made up of 2 coiled-coil forming regions, separated by a flexible proline-glycine ‘hinge’ that allows the kinesin dimer stalk to bend. Kinesin can exist in either an extended conformation or folded with its head and tail domains in close proximity (Hisanaga et al., 1989; Hackney et al., 1992). The conformation states of kinesin have an autoregulatory function; when folded, the C-terminal tail inhibits the N-terminal head and inhibits its ATPase activity (Coy et al., 1999). Cargo binding may relieve the tail-head inhibition and activate the motor (Friedman and Vale, 1999). A recent kinesin-1 motor-tail crystal structure suggests that the tail forms cross links at two positions with the motor domains that inhibit stepping (Kaan et al., 2011).
Figure 1-7. Figure showing the general structure of kinesin-1. The Khc motor domains at the N-terminus are represented in purple here, and are separated from the C-terminal tail domains (magenta) by a ~70nm stalk (grey). The kinesin light chains (in green) mediate cargo binding and, with the Khc tails, regulates the motor domains. The tetratricopeptide (TPR) motifs on the light chain interact with proteins such as JIPs (Jun-N-terminal kinase (JNK) interacting proteins). Figure adapted from (Vale, 2003).
Functions: Kinesin-1 is ubiquitously expressed and is essential for viability (Saxton et al., 1991). Its expression pattern suggests that kinesin-1 has important functions in all cells. The first in vivo functional studies were done in *Drosophila*. *Kinesin heavy chain (Khc)* mutations resulted in larval lethality, abnormal growth and distal paralysis (Saxton, 1991). In contrast to *Drosophila*, which has only one Khc, there are three Khc subtypes (Kif-1A, -1B and -1C) in mammals. Although highly similar, these isoforms differ in their expression patterns. Kif-1A and -1C are expressed only in neurons and Kif-1B is expressed in all tissues (Kanai, 2000). *Kif-1A* knockout mice die shortly after birth due to lung inflation abnormalities. *Kif-1B* knockout mice do not survive past 11.5 days post-coitum (Xia, 2003; Tanaka, 1998). *Kif-1C* knockout mice appear normal except for a small decrease in brain size and motor neuron numbers (Kanai, 2000).

Kinesin-1 plays essential roles in cytoplasmic organization during all stages of development such as in ooplasmic streaming, mRNA localization, axonal transport, and nuclear positioning in skeletal muscle (Hurd and Saxton, 1996; Brendza et al., 1999; Brendza et al., 2002; Pilling et al., 2006 Serbus, 2005; Metzger 2012; Wang 2013). Studies using electron microscopy and immunofluorescence localization indicated that kinesin-1 associates with membrane bound organelles (Hollenbeck, 1989; Leopold et al., 1992). Also, perfusion of squid axoplasm with antibodies to kinesin-1, or mutation of *Khc* disrupted axonal organelle movements and distribution (Brady et al., 1990; Hurd and Saxton, 1996). Kinesin-1 is now known to be the
anterograde motor for mitochondria (Pilling et al., 2006), peroxisomes (Kural et al., 2005), synaptic vesicle precursors (Pilling, unpublished data), neurofilaments (Uchida et al., 2009) and mRNPs (Ling et al., 2004). In addition to axonal transport, kinesin-1 also contributes to the movement of other cytoplasmic components. These include Golgi-ER trafficking (Lippincott-Schwartz, 1995; Gupta, 2008) and lysosome transport (Nakata, Hirokawa, 1995). More recently, kinesin-1 was shown to be important at synaptic terminals, where it mediates the delivery, removal and distribution of AMPA receptors (Frédéric J. Hoerndli, 2013). Clearly, kinesin-1 has important roles in the organization of the cytoplasm in many cell types and in many cellular processes.
<table>
<thead>
<tr>
<th>Standardized name</th>
<th>Representative family members</th>
<th>Reported function/structural features</th>
<th>Member no.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinesin-1</td>
<td>KIF5B, KHC, NKin, DdK3, DdK5</td>
<td>Vesicle transport, conventional</td>
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<td>Kinesin-2</td>
<td>KIF3A/3B, KIF17, Krp85/95, Osm3, Fla10</td>
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<td>Kinesin-3</td>
<td>KIF1A, KIF1B, KIF13A, UNC104, DdUnc104</td>
<td>Organelle transport/monomeric</td>
<td>8/4/2/0/1</td>
<td></td>
</tr>
<tr>
<td>Kinesin-4</td>
<td>KIF4A, KIF21A/B, Chromokinesin</td>
<td>Organelle transport, chromosome movement</td>
<td>5/3/2/3/1</td>
<td></td>
</tr>
<tr>
<td>Kinesin-5</td>
<td>KIF11, Eg5, BimC, CIN8, KIP1, Cut7</td>
<td>Spindle formation/homotetrameric, bipolar</td>
<td>1/1/1/4</td>
<td></td>
</tr>
<tr>
<td>Kinesin-6</td>
<td>KIF20, KIF23, Rab6Kinesin, CHO1, MKLP1</td>
<td>Cytokinesis, spindle polarity</td>
<td>5/2/1/0/1</td>
<td></td>
</tr>
<tr>
<td>Kinesin-7</td>
<td>KIF10, CENP-E, CMET, CANA, KIP2</td>
<td>Kinetochore microtubule capture</td>
<td>1/2/0/14/2</td>
<td></td>
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<tr>
<td>Kinesin-8</td>
<td>KIF18B, KIF19A, KLP67A, KIP3</td>
<td>Nuclear migration, mitochondrial transport</td>
<td>3/2/1/2/0</td>
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<tr>
<td>Kinesin-9</td>
<td>KIF6, KIF9, KRP3, CrKLP1</td>
<td>Unclear</td>
<td>2/0/0/0/0</td>
<td></td>
</tr>
<tr>
<td>Kinesin-10</td>
<td>KIF22, KID, Nod</td>
<td>Chromosome segregation/helix–hairpin–helix DNA-binding motif</td>
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</tr>
<tr>
<td>Kinesin-11</td>
<td>KIF26A, KIF26B, VAB8, SMY1</td>
<td>Signal transduction/divergent catalytic core</td>
<td>2/1/1/2/0</td>
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<tr>
<td>Kinesin-12</td>
<td>KIF12, KIF15, HKLP2, KLP54D, Xklp2, PAKRpd</td>
<td>Organelle transport/homologous tail</td>
<td>2/1/0/6/0</td>
<td></td>
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<tr>
<td>Kinesin-13</td>
<td>KIF2A, MC kak, XKCM1, PKinkl</td>
<td>Microtubule depolymerizing/central motor</td>
<td>4/3/2/1/1</td>
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<tr>
<td>Kinesin-14A</td>
<td>KIFC1, CHO2, Nod, Kar3, KatA</td>
<td>Chromosome segregation/ C-terminal motor,</td>
<td>1/1/4/1</td>
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<tr>
<td>Kinesin-14B</td>
<td>KIFC2, KIFC3, KatD, KCBP, KIF25</td>
<td>Organelle transport/C-terminal motor</td>
<td>3/0/1/16/0</td>
<td></td>
</tr>
<tr>
<td>Orphans</td>
<td>CeKLP10, CsKLP18, DdK9</td>
<td>Ungrouped</td>
<td>0/0/2/2/1</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Kinesin superfamily proteins and some of their reported functions. 

b shows the number of kinesin members in Human/ Drosophila/ C. elegans/ Arabidopsis/ Dicyostelium genomes. Table adapted from (Miki et al., 2005).
1.4 CURRENT QUESTIONS ABOUT THE MECHANISMS OF AXONAL TRANSPORT

Despite the wealth of information we have on motor proteins that drive transport, we are still in the process of understanding the basic mechanisms underlying axonal transport. How is transport spatially and temporally regulated? Determination of the contributions of motors to axonal transport is complicated by the diversity of motors, their largely unknown cargo interaction mechanisms, and functional interactions between different motors on the same cargo. For example, mitochondria transport is highly saltatory, their movements are frequently interrupted with pauses and their destinations along the axon can be influenced by local signaling from growth factors, ATP levels, and local calcium concentrations (Overly et al., 1996; Pilling et al., 2006; Wang and Schwarz, 2009). To circumvent the complexities associated with mitochondria, this thesis is focused on defining the transport characteristics of a relatively simple axonal cargo, dense core vesicles (DCVs). In contrast to mitochondria, DCVs are a well-defined type of vesicular organelle with a single axonal transport destination, the synaptic terminal. DCVs therefore offer an excellent opportunity to study a physiologically important but relatively simple transport process. The following section will briefly survey the current literature on the biogenesis, characteristics and transport of DCVs.
1.5 DENSE CORE VESICLES

Neurons and endocrine cells are dependent on secretion for cell-cell communication. A regulated secretory pathway transports neuropeptides in DCVs to the cell surface for secretion triggered by some stimulus. Endocrine organs use DCVs to regulate many key physiological processes including growth, appetite, mood, stress and cognition. (Guan 1997; Pacak & Palkovits 2001; Stanley et al 2005; Kenna et al 2009). In neurons, vesicles in the regulated secretory pathway include both DCVs and neurotransmitter-filled synaptic vesicles (SV). Together the contents released by DCVs and SVs mediate and modulate neurotransmission. Because of their roles in neurotransmission and endocrine function, the abnormal biogenesis, trafficking or secretion of DCVs and SVs can result in severe debilitating disorders (Gondre-Lewis et al., 2012).

The formation of DCVs is a multistep process that is similar in neurons and endocrine cells. In the nervous system, DCVs contain small neuropeptides and hormones such as endorphins and neurotrophic growth factors (BDNF, NGF and somatostatin). Similar to peptides and hormones secreted by other cell types, neuropeptides are synthesized in a precursor form (pre-pro-neuropeptide) and are subsequently cleaved to the active form. Analogous to alternative mRNA splicing, the cleavage site targeted by the processing enzymes can give rise to several different
active peptides. In humans, there are ~100s of neuropeptides encoded by approximately 90 different genes (reviewed by (Gondre-Lewis et al., 2012)).

A highly regulated, post-translational process orchestrates the packing of peptides in their respective vesicles. Secretory proteins are synthesized at the rough ER and are then directed to the Golgi apparatus where they undergo post-translational modifications and proteolytic cleavage. It is at that point that neuropeptide precursors are sorted into their respective vesicles. A detailed understanding of the sorting mechanism for DCV formation is still lacking but it is clear that sorting involves aggregation in the TGN at an acidic pH in a calcium dependent manner. After sorting, membrane budding forms immature DCVs (Dannies, 2012; Orci et al, 1987; Hutton 1983). DCVs then mature by acidification to pH ~5.5 and precursor peptides are processed into active forms. Mis-sorted proteins and proteins no longer required by DCVs are removed by a clathrin-dependent process (Tooze and Tooze 1986; Teuchert 1999). Mature DCVs are stored in “reserve pools” and upon external stimulation, Ca2+ influx triggers the secretion of DCV peptides into the extracellular environment (Orci, 1987) (Figure 1-8).

The movement of newly formed DCVs from the TGN to the cell cortex for secretion is largely dependent on microtubule based transport (Rudolf et al., 2001; Alexander et al., 2005). DCVs are transported to distal release sites along microtubules and then are transferred and docked onto cortical actin filaments. There
is debate as to which motors are required for DCV movement along microtubules. In beta cells, the transport of insulin-filled DCVs to the cell periphery is kinesin-1 dependent; inhibition of kinesin-1 with RNAi led to a loss of long-term glucose stimulated release of insulin (Varadi et al., 2002; Varadi et al., 2003). In contrast, in *C.elegans, Drosophila* and cultured hippocampal neurons, kinesin-3 is a primary anterograde motor for the axonal transport of DCVs (Zahn 2004; Barkus, 2008; Lo, 2011). In these studies, function disruption of kinesin-3 by genetic approaches or RNAi dramatically reduced the number of mobile DCVs. The differences between these studies could be cell type dependent, but the use of different anterograde motors points to the possibility that two or more kinesin family members may transport DCVs.

As described earlier, DCVs are essential organelles responsible for a myriad of cellular and physiological processes. However the mechanism underlying the regulation and distribution of neuronal DCVs remains unknown. Before we can further our understanding on DCV transport, we have to first identify all the force producing components responsible for DCV movement. Chapter 2 describes the identification of motor proteins essential for DCV transport in *Drosophila* axons. Chapter 3 describes ongoing work on the consequences axonal DCV transport defects and how they relate to neurodegeneration.
Two distinct secretory pathways are present in neuroendocrine and endocrine cells. Constitutive (left) and regulated (right) secretion pathways are illustrated. DCVs belong to the regulated secretion pathway (right). Figure modified from (Kim et al., 2006).
CHAPTER 2 : MOTOR PROTEINS IN AXONAL TRANSPORT OF NEUROPEPTIDE VESICLES

2.1 INTRODUCTION

Because the biogenesis of many RNAs, proteins, and organelles occurs in or near the cell body, neurons rely heavily on long-distance microtubule-based transport to build and maintain their axons. The transport is bidirectional, driving regulated streams of fresh components toward synaptic terminals (anterograde) and of aged or endocytosed materials back toward the cell body (retrograde) (Brown, 2013). There is intense interest in axonal transport, both because it offers paradigms for cytoplasmic transport mechanisms in all cell types, and because defects in transport mechanisms have been shown to cause synaptic transmission inhibition, axon degeneration, paralysis, and death (Gho et al., 1992; Hurd and Saxton, 1996; Martin et al., 1999; Hafezparast et al., 2003; Puls et al., 2003; Crimella et al., 2012) (reviewed by De Vos et al., 2008; Morfini et al., 2009; Perlson et al., 2010).

Motor proteins create force that drives bidirectional transport along microtubules. Anterograde movement is driven by plus-end directed N-terminal kinesins and retrograde movement is driven by minus-end directed dyneins (Franker and Hoogenraad, 2013). There are at least 14 different kinesin families (Lawrence et al., 2004; Wickstead and Gull, 2006; Hirokawa et al., 2010) but in axons, it appears
that most anterograde transport is driven by kinesin-1, -2 or -3 heavy chains complexed with various cargo-specific linkers and regulators (Goldstein et al., 2008). Cytoplasmic dynein heavy chain-1 (DYNC1H1: referred to as dynein below) is the most prominent minus-end motor. It too has a variety of associated proteins that are thought to serve as cargo-specific linkers and regulators (Schiavo et al., 2013).

The transport characteristics of cellular cargoes are largely dictated by the combined actions of multiple motors attached to the cargoes. The net transport direction is the result of multiple motors of opposite polarity (anterograde vs retrograde motors) and is also dependent on the overall forces generated by motors of similar polarity (such as multiple copies of the same or different kinesins). Studies in vitro and in vivo agree that having multiple motors of the same polarity on a cargo increases its run length but has negligible effects on its run velocity (Howard, 1989; Derr, 2012; Shubeita, 2008). However, motors with different run velocities can cooperate to produce intermediate velocities (Ou, 2005; Pan, 2006; Larson, 2008). With regards to motors of opposite polarities, it is apparent that opposing anterograde and retrograde motors are often present on the same organelle (Welte et al., 1998; Gross et al., 2000; Ligon et al., 2004; Soppina et al., 2009; Encalada et al., 2011). Tests of various transport processes indicate that opposing motors on an organelle usually have strong positive influences on one another, rather than antagonistic influences (Brady et al., 1990; Martin et al., 1999; Ling et al., 2004; Pilling et al., 2006; Barkus et al., 2008; Ally et al., 2009; Uchida et al., 2009). Two hypotheses for
this surprising interdependence have been suggested: 1) opposing motors have biochemical relationships that activate one another and perhaps also coordinate them to avoid antagonism (Martin et al., 1999; Deacon et al., 2003; Ligon et al., 2004; Kwinter et al., 2009; Fu and Holzbaur, 2013) or 2) motors have biophysical relationships such that force generation by a motor or team of motors is activated by strain created by a weaker opposing motor on the same cargo (Ally et al., 2009; Welte, 2010).

The definition of axonal transport mechanisms, beyond the common need for microtubules and motors, remains challenging. This is in large part due to the diversity of cargos and their potential motors, to complex regulatory control of delivery rates and destinations, and to the unsolved puzzles of motor-motor interdependence (Schlager and Hoogenraad, 2009; Brown, 2013). To gain new insights, we have focused on the characterization of a single organelle, neuropeptide-filled dense core vesicles (DCVs), in the axons of motor neurons in living Drosophila. DCV behavior is simple with resolute fast movement between the two endpoints of the transport path; the cell body and synaptic terminals. It is also robust; persisting through harsh treatment of the animal. Using function inhibition and time-lapse imaging of motor axons, our results indicate that kinesin-1, kinesin-3, and dynein are the motors for long-distance DCV transport and exhibit unique motor-motor interdependence. We find that two different anterograde motors, kinesin-1 and kinesin-3 are functionally dependent on each other for DCV transport. We discuss a
model in which kinesin-3 (Unc-104) carries DCVs from sites of biosynthesis in the neuronal cell body into the initial segment of the axon where it and kinesin-1 (Khc) cooperate to drive fast processive runs toward the synaptic terminal. Similar to previous studies on motor interdependence, we find that anterograde process requires dynein as an activator. Dynein carries unused DCVs retrograde in a process activated differentially by kinesin-1 and -3. The distinct activation influences of the two kinesins cannot be explained by simple biophysical strain, thus favoring a biochemical activation mechanism.
2.2 MATERIALS AND METHODS

_Drosophila strains._

_Drosophila_ were cultured with 12hr light-dark cycles at 22-25°C, according to standard protocols (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). The transgenes \( P[w^{+mC} \text{UAS-GFPmito}]AP.3 \) (Pilling et al., 2006) and \( P[w^{+mC} \text{UAS-ANFGFP}]3 \) (Rao et al., 2001) were expressed in motor neurons using Gal4 “driver” transgenes (either \( P[\text{GawB}]D42 \) or \( P[\text{GawB}]VGlut\text{OK371} \)) to load GFP into the mitochondria matrix or the DCV lumen respectively. For biochemical fractionation, \( P[w^{+mC} \text{UAS-ANFGFP}]3 \) was expressed using \( P[\text{tubP}-\text{GAL4}]LL7 \). Kinesin-1 mutants were constructed using hypomorphic point mutations (\( \text{Khc}^6 \), or \( \text{Khc}^{17} \)) with a null (\( \text{Khc}^{27} \)) (Brendza et al., 1999). Hypomorphic dynein mutants were constructed using \( \text{Dhc64C}^{6-10} \) and \( \text{Dhc64C}^{4-19} \) (Gepner et al., 1996). For RNAi, fly strains carrying Gal4-UAS controlled transgenes capable of expressing hairpin RNAs specific for Khc (\( P[\text{TRiP.GL00330}]\text{attP2} \)), for Dhc64C (\( P[\text{TRiP.HMS01587}]\text{attP2} \), and for Milton (\( P[\text{TRiP.JF03022}]\text{attP2} \)) were obtained from the Transgenic RNAi Project (TriP. Harvard University, http://www.flyrnai.org/TRiP-HOME.html). To enhance the efficacy of Milton RNAi, Dicer was co-expressed from the \( P[w^{#+mC}]=\text{UAS-Dcr-2.D}]1 \) transgene.
**Immunofluorescence imaging.**

Neuromuscular systems were prepared from wandering 3rd instar larvae, fixed, and stained as described previously (Hurd and Saxton, 1996). Mouse monoclonal anti-cysteine string protein (CSP) was used at 1:500 (dCSP-2(6D6)-c; Drosophila Hybridoma Bank, University of Iowa). Alexa coupled anti-mouse secondary was used at 1:1000 (Invitrogen, Carlsbad, CA). Stained larvae were mounted on slides in VectaShield (Vector Laboratories). Imaging was done with an inverted spinning disk confocal fluorescence microscope (Nikon/Perkin Elmer/Improvision Ultraview) equipped with a Hamamatsu C9100-50 EM CCD camera. Nerve terminals at muscle pairs 6/7 and 12/13 were imaged in segments 4-6. Control and mutant preparations were fixed and stained using identical procedures and were imaged using identical acquisition settings.

**Live imaging.**

Time-lapse imaging of GFP-loaded organelles in axons was done with living third instar larvae 4-5 days after egg lay. A larva was placed in an imaging chamber modified from Fuger et al. (Fuger et al., 2007) and anesthetized by injecting 50ul of chilled Desflurane (Baxter) into the chamber, then sealing it. For each animal, segmental nerve 7 or 8 passing through segments A4-A5 was imaged through the ventral body wall with the spinning disk microscope using a 60X 1.4Na objective (Nikon). Images of DCVs and mitochondria were collected at 2 frames/sec and 1 frame/sec respectively for at least 500 frames. After imaging, larvae were returned to
normal culture medium. Data were analyzed only from larvae that survived the procedure and recovered crawling mobility. Although larvae can survive in the anesthesia chamber for hours, imaging was restricted to the initial 30min to minimize the potential for physiological stress.

**Fractionation of Drosophila cytoplasm and western blotting.**

To fractionate DCVs, 2g of adult flies with ANF::GFP expression driven by the $P^{\#}$tubP-GAL4/LL7 driver were homogenized on ice using a mortar and pestle in 10ml of homogenization buffer (250mM sucrose, 100mM K-acetate, 40mM KCl, 20mM HEPES, 10mM Tris, 5mM EGTA, 5mM MgCl$_2$, 1mM MgATP, 1 complete mini-EDTA free protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN), pH 7.4). Despite the ubiquitous expression pattern of $P^{\#}$tubP-GAL4/LL7 (O'Donnell et al., 1994), larval ANF::GFP expression was detected only in the nervous system, suggesting that the mRNA or protein is unstable in non-neuronal cells. To remove fly debris, homogenate was filtered through a nitex mesh (pore size=140µm) and then clarified by centrifugation twice at 1300 x g for 5min. Most mitochondria were then eliminated by centrifugation for 10min at 5000 x g. Post mitochondrial supernatants from two preparations were pooled and DCVs were pelleted by centrifugation at 100,000 x g for 20min. The pellet was resuspended in 1ml homogenization buffer, overlaid on a 20-60% sucrose gradient prepared on The Gradient Station (Biocomp Instruments), and centrifuged at 134,000 x g for 90min in a swinging bucket rotor.
Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and western blotting. After blocking in Tris-buffered saline/Tween20 with 5% non-fat milk, blots were incubated with primary antibodies diluted in blocking solution: rabbit anti-Imac/Unc-104 1:1000 (Pack-Chung et al., 2007), rabbit anti-
*Drosophila* Khc 1:1000 (Cytoskeleton), mouse anti-GFP 1:1000 (Clonetech), mouse anti-cytochrome c 1:100 (Neomarkers). Blots were washed in blocking solution and incubated with secondary antibodies: horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000) and HRP-conjugated goat anti-mouse IgG (1:10,000) (Jackson ImmunoResearch Laboratories) diluted in blocking solution. Secondary antibodies were detected using chemiluminescence (GE Healthcare).

**Flux, particle tracking, and statistics.**

To measure overall transport of ANF-GFP DCVs from time-lapse image series, a line was drawn perpendicular to one nerve and the number of particles passing that line in each direction was counted for 200 frames. The counts were divided by time to derive anterograde and retrograde flux values. A Student's t-test was used to distinguish significant flux differences between control and mutant genotypes.

Individual DCV tracking was done using Image J 1.38x with the Manual Tracker plugin (Rasband, 1997). Automated tracking was not effective due to the high density of DCVs in nerves. In pilot tests with data from tracking all organelles
in many time-lapse series, we previously determined that a sample size of 5 particles in each direction from each of 10 animals (one nerve each) provides a robust statistical analysis that is not substantially changed by increased sample sizes (Pilling et al., 2006; Barkus et al., 2008). In the present study, 5 anterograde and 5 retrograde particles were tracked per nerve for as long as each particle remained in the plane of focus or in the field of view. Position-time data were analyzed as described previously (Pilling et al., 2006). Each particle track was parsed into 2 states: runs or pauses. A run was defined as a period of continuous motion for a minimum of 3 frames. Because DCV movement was highly processive in controls, pauses were rare and measured run distances were often limited by the field of view. Thus, our mean run lengths for controls are underestimates. Run velocity is derived from run distance divided by time. Duty cycle refers to the fraction of time a particle spends in runs or pauses over its total tracked duration. A run in the primary direction for a particular DCV is a forward run, i.e. an anterograde DCV in the anterograde direction or a retrograde DCV in the retrograde direction. Because DCVs showed very few reverse runs, reverse run data were excluded in our duty cycle analyses. To determine if a single transport parameter was significantly different due to genotype, linear contrast analysis was carried out using SPSS Version 18.0 (SPSS Inc., Chicago) (Pilling et al., 2006). All data presented here represent means and standard errors.
2.3 RESULTS

2.3.1 Kinesin-1 is required for DCV distribution and transport in neurons

To identify microtubule motors that contribute to DCV transport, we used RNAi to test candidate kinesins in *Drosophila* larvae. RNAi transgenes specific for either *Khc* (kinesin-1), *Klp64D* (kinesin-2), or *Unc-104* (kinesin-3) transcripts were expressed in neurons along with a neuropeptide-GFP fusion protein (ANF::GFP) that is targeted to the lumens of DCVs (Rao et al., 2001; Barkus et al., 2008; Djagaeva et al., 2013). *Klp64D* RNAi caused no discernable changes in larval behavior or DCV distribution. In contrast, *Unc-104* RNAi caused lethality and a decrease in the amount of ANF::GFP in axons, and an increase in the amount of ANF::GFP in the ventral ganglion, where motor neuron cell bodies reside. This is consistent with previous studies that identified kinesin-3 as an important anterograde motor for DCVs in neurons (Zahn et al., 2004; Barkus et al., 2008; Lo et al., 2011). *Khc* RNAi in motor neurons caused distal paralysis ("tail flipping"), lethality, and an accumulation of ANF::GFP in nerves, suggesting that kinesin-1 is also important in axonal DCV transport. To examine this further, *Khc*\(^6\), a well characterized hypomorphic missense allele that changes Coil 2 of the Khc stalk, was combined with a nonsense null allele (*Khc*\(^{27}\)). Examined in dissected neuromuscular preparations, ANF::GFP in mutants was concentrated in axonal focal accumulations (Figure 2-1A) and was abnormally low in axon terminal boutons (Figure 2-1A). These observations confirmed that kinesin-1 influences axonal DCV distribution.
To determine if kinesin-1 influences DCV movement, ANF::GFP in the axons of live anesthetized larvae was imaged using time-lapse confocal microscopy and flux was quantified (Figure 2-1C, D Table 2, Movies 1 and 2 – see Supplemental Files). $Khc^{6}/Khc^{27}$ caused a 6-fold reduction in anterograde flux ($5.9 \pm 1.3 \text{DCV/min vs } 35.7 \pm 4.6 \text{ DCV/min in control}$) and a 14-fold reduction in retrograde flux ($0.7 \pm 0.2 \text{ DCV/min vs } 10 \pm 2.5 \text{ DCV/min in control}$). $Khc^{17}$, a missense allele that changes Loop 11 of the motor domain (Brendza et al., 1999), when placed over the null, caused 2-fold and 6-fold reductions in anterograde and retrograde flux, respectively (Table 2). A $Khc$ RNAi transgene expressed specifically in motor neurons allowed almost no flux in either direction. These results show that kinesin-1 is required for anterograde transport of axonal DCVs. The effect on retrograde DCV return is interesting but not surprising, since interdependence of anterograde and retrograde transport has been reported for a variety of processes (Martin et al., 1999; Ling et al., 2004; Pilling et al., 2006; Barkus et al., 2008; Uchida et al., 2009; Encalada et al., 2011).

To examine the effects of Khc inhibition on run behavior, DCVs that moved in Khc mutant nerves were tracked individually. In wild-type axons, forward runs were long and reverse runs were rare. Thus we focused on run-pause duty cycles, forward run velocities, and forward run lengths (Figure 2-2Table 2). Anterograde DCVs in $Khc$ mutants spent 3-fold more time in pauses ($15 \pm 5 \text{ vs } 5 \pm 3\%$ for control),
and the average length of runs was reduced 3-fold (6.2±2.1 vs 19.1±1.5 µm for control). Anterograde run velocity in Khc mutants was significantly reduced (0.67±0.09 vs 0.96±0.03µm/s). These effects on specific anterograde DCV transport parameters are consistent with kinesin-1 as an anterograde axonal transport motor for DCVs.
<table>
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<tr>
<th>Genotype</th>
<th>Larva&lt;sup&gt;Ψ&lt;/sup&gt; (runs)</th>
<th>Flux (DCV/min)</th>
<th>Run Length (µm)</th>
<th>Run Velocity (µm/s)</th>
<th>Fwd Run (%)</th>
<th>Pause (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (for Khc)</td>
<td>10 (95)</td>
<td>35.72±4.64</td>
<td>19.1±4.74</td>
<td>0.96±0.1</td>
<td>94±3.5</td>
<td>5.0±3.2</td>
</tr>
<tr>
<td><em>Khe&lt;sup&gt;6&lt;/sup&gt;/Khc&lt;sup&gt;27&lt;/sup&gt;</em></td>
<td>10 (174)</td>
<td>5.9±1.3</td>
<td>6.2±2.09</td>
<td>**</td>
<td>85±5.7</td>
<td>15±5.4</td>
</tr>
<tr>
<td><em>Khe&lt;sup&gt;17&lt;/sup&gt;/Khc&lt;sup&gt;27&lt;/sup&gt;</em></td>
<td>10 (182)</td>
<td>16.5±2.01</td>
<td>10.4±3.73</td>
<td>***</td>
<td>86±5.1</td>
<td>14±5.1</td>
</tr>
<tr>
<td>Control (for Dhc)</td>
<td>10 (108)</td>
<td>50.47±3.49</td>
<td>20.9±4.46</td>
<td>1.02±0.09</td>
<td>87±6.8</td>
<td>13±6.8</td>
</tr>
<tr>
<td><em>cDhc64C&lt;sup&gt;610&lt;/sup&gt;/cDhc64C&lt;sup&gt;4-19&lt;/sup&gt;</em></td>
<td>7 (86)</td>
<td>9.6±1.41</td>
<td>11.3±4.04</td>
<td>***</td>
<td>97±2.8</td>
<td>3.0±2.2</td>
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</table>

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<tr>
<th>Genotype</th>
<th>Larva&lt;sup&gt;Ψ&lt;/sup&gt; (runs)</th>
<th>Flux (DCV/min)</th>
<th>Run Length (µm)</th>
<th>Run Velocity (µm/s)</th>
<th>Fwd Run (%)</th>
<th>Pause (%)</th>
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<tbody>
<tr>
<td>Control (for Khc)</td>
<td>10 (161)</td>
<td>10.08±2.49</td>
<td>-8±2.34</td>
<td>-0.97±0.1</td>
<td>87±3.8</td>
<td>11±3.2</td>
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<tr>
<td><em>Khe&lt;sup&gt;6&lt;/sup&gt;/Khc&lt;sup&gt;27&lt;/sup&gt;</em></td>
<td>7 (50)</td>
<td>0.7±0.24</td>
<td>**</td>
<td>-5.5±1.49</td>
<td>80±8.9</td>
<td>18±7.9</td>
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<tr>
<td><em>Khe&lt;sup&gt;17&lt;/sup&gt;/Khc&lt;sup&gt;27&lt;/sup&gt;</em></td>
<td>9 (94)</td>
<td>1.56±0.62</td>
<td>**</td>
<td>-9.7±3.67</td>
<td>80±7.3</td>
<td>15±5.4</td>
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<tr>
<td>Control (for Dhc)</td>
<td>10 (155)</td>
<td>18.85±2.28</td>
<td>-8.2±2.66</td>
<td>-0.89±0.1</td>
<td>66±11</td>
<td>31±9.8</td>
</tr>
<tr>
<td><em>cDhc64C&lt;sup&gt;6-10&lt;/sup&gt;/cDhc64C&lt;sup&gt;4-19&lt;/sup&gt;</em></td>
<td>5 (61)</td>
<td>0.84±0.45</td>
<td>**</td>
<td>-2.4±0.83</td>
<td>87±4.4</td>
<td>10±3.5</td>
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</table>

Table 2 DCV transport parameters in Drosophila axons. All values represent means (± SE). Statistical comparison of flux values was done using a Student's t-test. All other comparisons were done by linear contrast. (* p < 0.05, ** p < 0.01, *** p < 0.001). <sup>Ψ</sup> Refers to the number of animals analyzed for each genotype and the total number of runs are indicated in brackets.
Figure 2-1 Kinesin-1 influences the distribution and flux of axonal DCVs. ANF::GFP, which concentrates in the lumens of DCVs, was expressed in neurons of control and Khc mutant (Khc\(^6\)/Khc\(^27\)) larvae using the P[GawB]D42 driver.

A) Confocal images of fixed control and Khc mutant segmental nerves in segments A4-5 showing ANF::GFP (green) and CSP (red), which is a vesicle associated synaptic terminal protein. Note the shift from evenly distributed punctate fluorescence in the control to focal axon accumulations in the mutant.
B) Synaptic terminals on muscles 6/7 of control or Khc mutant larvae. Scale bar = 12µm.

B’) Higher magnifications of the boxed areas in (B) showing just ANF::GFP signal. Note the reduced presence of ANF::GFP in the boutons of the Khc mutant (right). Scale bar = 3µm.

C) Representative kymographs generated from 100sec of time lapse recording (2 frames/sec) of control and Khc mutant segmental nerves in live anesthetized larvae showing ANF::GFP position (x-axis) as a function of time (y-axis). Negative slopes, positive slopes and vertical lines indicate anterograde, retrograde and stationary DCVs respectively. Note the reduced number of anterograde and retrograde DCV tracks in the Khc mutant. Scale bar = 5µm.

D) Quantification of flux, which is the number of DCVs moving past a given point in one segmental nerve per minute. Bars show mean (± SE) for n=10 animals per genotype (1 nerve per animal). Brackets show significant differences between Khc mutant and control values determined by a Student's t-test (** p < 0.001).
Figure 2-2 Kinesin-1 influences anterograde DCV run behaviors. ANF::GFP was expressed in neurons of control and Khc mutant (Khc^6/Khc^27) larvae using the P[GawB]D42 driver. DCVs in axons of segmental nerves were imaged in live animals, then individually tracked, and run behaviors were quantified.

A) DCV transport duty cycles, shown as the mean percent time (± SE) spent in either runs or pauses for control and Khc mutants. n=10 animals per genotype (1 nerve per animal).

B) Frequency histograms showing run lengths (top, bin size=1µm) and run velocities (bottom, bin size=0.1µm/s) in axons of Khc mutant and control larvae. Arrows indicate means. n = 10 larvae for each genotype (1 nerve per animal). Note the shift to shorter run lengths and slower velocities for anterograde DCVs in Khc mutants.
Brackets show significant differences as determined by linear contrast (**p ≤ 0.01, *** p ≤ 0.001).
2.3.2 Evidence for a direct Kinesin-1 DCV relationship

Kinesin-1, well known as a motor for mitochondria (Pilling et al., 2006), has not been reported as a motor for axonal DCVs. To determine if the influence of Khc inhibition on DCVs could be due to defects in axonal mitochondria distribution and thus in the availability of ATP from oxidative phosphorylation, we used RNAi to inhibit Milton a mitochondria-Khc linker protein (Schwarz, 2013). Consistent with previously reported zygotic Milton mutant studies (Glater et al., 2006), motor neuron-specific Milton RNAi severely reduced the number of mitochondria in distal regions of the axons, far from the cell bodies contained within the ventral ganglion. Mitochondria were observed in axons near the ventral ganglion (segments A2-3), but not more distally, and transport was minimal (Figure 2-3A, Movies 3 and 4 see Supplemental Files). In contrast, axonal DCV distribution, anterograde flux, and transport behavior was robust, including in distal segments (Figure 2-3 B, C, Movies 5 and 6 see Supplemental Files). This shows that the effects of Khc mutations on anterograde DCV transport are not due to defects in mitochondria distribution.

Our Milton inhibition data raise two additional issues. First, Milton RNAi reduced retrograde DCV flux by a factor of 2. This is a mild effect relative to those caused by Khc inhibition, but it indicates that Milton and/or mitochondria distribution influences the number of DCVs that can move retrograde. This could be due to inhibition of the retrograde transport machinery or to increased release of...
neuropeptides from terminals leaving fewer DCVs available for retrograde transport. The second puzzle comes from the observation that Milton RNAi severely limits mitochondria presence in distal axons, yet anterograde DCV transport there is normal. What is the source of energy for anterograde DCV movement in distal axons? Recent studies showed that small vesicle transport in axons uses vesicle associated glycolysis as the main source of energy (Zala et al., 2013), so perhaps anterograde DCV motors likewise rely on glycolysis as a source of ATP.

If kinesin-1 is a motor for DCVs, it must physically associate with them. To test this, we generated lysates of flies expressing ANF::GFP and looked for co-fractionation of Khc with DCVs during sucrose gradient sedimentation. Western blots show a peak of Khc that co-fractionated with ANF::GFP and with Unc-104, the previously identified anterograde DCV motor (Figure 2-4). Cytochrome C was not detected in that peak, indicating that the presence of Khc was not due to contamination by mitochondria. Overall, our results indicate a direct role for kinesin-1 as an anterograde DCV motor.
Figure 2-3 Milton does not influence anterograde DCV run behavior.
Milton RNAi was induced specifically in motor neurons of larvae expressing either mito-GFP or ANF::GFP using the P[GawB]VGlut\textsuperscript{OK371} motor neuron driver. Larvae were anesthetized and fluorescence in segmental nerves was imaged by time lapse microscopy.

A) Kymograph generated from a 200sec time lapse recording (1 frame/sec) of mito-GFP showing mitochondria transport in axons of control or Milton RNAi (Milt) larvae in nerves near the ventral ganglion which contains the motor neuron cell bodies segments (A2-3). Note that Milton RNAi eliminated most mitochondrial movement. Few or no axonal mitochondria were seen in nerves more distally.

B) Kymographs generated from a 100sec time lapse recording (2 frames/sec) showing ANF::GFP transport in axons of control or Milton RNAi larvae in distal segments A5-6. Scale bars = 5\(\mu\)m.

C) Quantification of DCV flux in control (n=6) and Milton RNAi (n=8) larvae (1 nerve per animal) in segments A5-6. The 2-fold decrease in retrograde DCV flux was marginally significant (Student's t-test, * p \leq 0.05).
Figure 2-4 Co-fractionation of kinesin-1 and kinesin-3 with DCVs. Homogenate of adult flies expressing ANF::GFP was fractionated by differential centrifugation and then by sucrose gradient sedimentation. Fractions were collected from the top of the gradient. A western blot shows the distribution of ANF::GFP in the gradient relative to a mitochondria marker (Cytochrome C), kinesin-1 (Khc) and kinesin-3 (Unc-104). Note that both kinesins co-fractionate with the ANF::GFP marker for DCVs.
2.3.3 Dynein is required for DCV transport in both directions

To test the influence of dynein on DCV distribution, we studied the effects of a hypomorphic dynein allele combination, \( Dhc64C^{6-10}/Dhc64C^{4-19} \) known to inhibit the transport of axonal mitochondria (Gepner et al., 1996; Pilling et al., 2006). Comparison of ANF::GFP fluorescence with anti-CSP staining in dynein mutant larval neuromuscular preparations revealed large focal accumulations of ANF::GFP in segmental nerve axons (Figure 2-5A) and an enrichment of ANF::GFP in the boutons of axon terminals (Figure 2-5B). These results are consistent with previously reported effects of dynein mutations on the distribution of axonal mitochondria, which are a known dynein cargo (Pilling et al., 2006). To determine if \( Dhc64C \) mutations alter DCV flux, time lapse imaging of ANF::GFP in axons was performed in anesthetized larvae (Figure 2-5C, D, Movies 7, 8 see Supplemental Files). Mutant nerves averaged a 22-fold reduction in retrograde flux (0.84±0.5 vs 18.9±2.3 DCV/min in control), and also a 5-fold reduction in anterograde flux (9.6±1.4 vs 50.5±3.5 DCV/min in control). Motor neuron Dhc64C RNAi had similar effects, indicating that these phenotypes reflect dynein function in neurons.
Figure 2-5 Influence of Dynein on the distribution and flux of DCVs. ANF::GFP was expressed in neurons of control and \( cDhc64C^{6-10}/cDhc64C^{4-19} \) mutant (Dhc) larvae using the \( P[GawB]D42 \) neuronal driver.
A) Confocal images of segmental nerves (n) and motor neuron terminal boutons (arrowheads) in segments A5-6 of fixed larvae showing ANF::GFP (green) and immunostaining for CSP (red). Note the shift from faint punctate fluorescence in the control to focal axon accumulations in the mutant nerves.

B) Another view of synaptic terminals (muscles 6/7). Note the increased ANF::GFP signal in mutant boutons. Scale bar = 12μm for A, B.

C) Kymographs generated from 100sec time lapse recordings (2 frames/sec) of ANF::GFP in control (top) and Dhc mutant (bottom) segmental nerves. The large vertical bands in the mutant reflect focal accumulations of ANF::GFP. Scale bar = 5μm.

D) Quantification of DCV flux in single nerves of control and Dhc mutants. Mean (± SE), n=10 control and 7 Dhc mutant animals (1 nerve per animal). Brackets show significant differences in both anterograde and retrograde flux determined by a Student's t-test (*** p ≤ 0.001).
To determine if dynein inhibition influences DCV run parameters, tracking analysis was performed (Figure 2-6Table 2). Retrograde DCVs in mutants spent 3-fold more time in pauses (30±10 vs. 10±3.5% in controls). The increased pausing correlated with substantially reduced retrograde run lengths (2.4± 0.83 vs.8.2± 2.7µm in control) and retrograde run velocities were reduced by 40% (0.51±0.11 vs. 0.89±0.09µm/s in control). These defects in specific retrograde transport parameters, combined with the dramatic retrograde flux inhibition and altered DCV distribution, indicate that dynein is the retrograde motor for axonal DCVs.

It is interesting that *Dhc64C* mutations also caused anterograde parameter changes (Figure 2-6Table 2). Anterograde DCVs in mutants spent 4-fold more time in pauses (13±6.8 vs. 2.6±2% in control), anterograde run lengths were reduced 2-fold (11.3±4.0 vs. 20.9±4.46µm in controls), and anterograde run velocities were reduced by 20% (0.81± 0.12 vs. 1.02± 0.09µm/s in controls). These changes are substantial as well as significant, indicating that dynein is important for kinesin-1 and/or kinesin-3 transport of anterograde axonal DCVs.
ANF::GFP was expressed in axons of motor neurons of control and cDhc64C6-10/cDhc64C4-19 mutant larvae using the P[GawB]D42 neuronal driver. DCVs in segmental nerves were imaged then individually tracked and their run behaviors were quantified.

A) DCV transport duty cycles, shown as the mean percent time (± SE) spent in either runs or pauses for control and cDhc64C mutants. Brackets show significant differences between control and mutant values as determined by linear contrast (**p < 0.001**).

B) Frequency histograms showing run lengths (bin size=1µm) and run velocities (bin size=0.1µm/s) from control and Dhc mutant larvae. Arrows indicate means. Brackets show significant differences between Dhc mutant and control values. n=10
control and 7 Dhc mutant animals (1 nerve per animal). *** p ≤ 0.001. Note the shift to shorter run lengths and slower velocities for both anterograde and retrograde DCVs in mutants.
2.4 DISCUSSION

Long distance movement of mRNAs, various vesicles, mitochondria and other organelles is crucial for the proper growth and function of axons. Transport mechanisms for different types of cargoes, beyond the shared use of microtubules as tracks, vary substantially and none are understood in detail. To help establish a detailed mechanism for a single axonal transport process, we have focused on the neuropeptide filled DCV, a physiologically important organelle that should use a relatively simple transport system. The expectation of simplicity stems from the observation that DCVs formed in the cell body have a single axonal transport destination; the presynaptic terminal (Gondre-Lewis et al., 2012) and thus should not need complex regulatory transport mechanisms. In keeping with this, anterograde DCVs in axons move rapidly via exceptionally long runs with few pauses (Zahn et al., 2004; Barkus et al., 2008; Lo et al., 2011).

Our results indicate that axonal DCVs employ three different microtubule motors. We previously identified Drosophila Unc-104, a kinesin-3, as an important anterograde motor (Barkus et al., 2008). Our work here identifies Khc, a classic kinesin-1, as a second anterograde motor, and cytoplasmic dynein as the retrograde motor. It is especially interesting that bidirectional transport flux defects are caused by inhibition of any one of these motors, raising important questions about why and how they influence one another's functions.
2.4.1 Opposing MT motor interdependence:

Studies of various transport phenomena have shown that inhibition of either kinesin-1 or dynein can stop transport in both directions, suggesting that anterograde and retrograde motors are functionally interdependent in some processes (Brady et al., 1990; Waterman-Storer et al., 1997; Martin et al., 1999; Ling et al., 2004; Pilling et al., 2006; Kim et al., 2007). On the other hand, inhibition of the anterograde melanosome motor, kinesin-2, does not impair retrograde transport by dynein (Gross et al., 2002), and inhibition of dynein actually facilitates kinesin-1 driven cytoplasmic streaming (Serbus et al., 2005). Perhaps some motility processes have evolved with a motor pairing strategy to optimize balanced bidirectional transport (Welte, 2010).

The mechanistic basis of opposing motor interdependence is a subject of ongoing debate. One possibility is that different types of motors have important biochemical associations, forming discrete assemblies in which opposing motors act on one another as allosteric activators (Welte et al., 1998; Shubeita et al., 2008; Welte, 2010). Biochemical associations between some subunits of opposing motors have been found (Deacon et al., 2003; Ligon et al., 2004; Kwinter et al., 2009; Fu and Holzbaur, 2013), but a complex that includes the heavy chains of dynein and kinesin-1 and/or kinesin-3 has not been reported. Perhaps such associations are especially labile and thus are difficult to isolate. Another possibility is that biochemical
associations are not important for interdependence; rather opposing motors activate one another biophysically. For example, full activity of forward motors requires strain from an opposing motor attached to the same cargo (Ally et al., 2009). This raises questions about how opposing motors avoid antagonistic paralysis, but theoretical studies suggest that efficient bidirectional transport could be accomplished by competing sets of motors, with productive motion in one direction determined simply by a moderate force advantage by one set or the other (Muller et al., 2008; Derr et al., 2012). Strong evidence of such a biophysical strain activated mechanism has been shown for peroxisomes in Drosophila S2 cultured cells, where activation of a microtubule motor can be accomplished by any opposing motor that is engineered for peroxisome attachment, regardless of its identity (Ally et al., 2009).

Our current and past results show that normal dynein-driven retrograde DCV transport requires two anterograde motors, kinesin-1 and kinesin-3. It is particularly interesting that kinesin-1 and -3 inhibitions caused different defects in retrograde transport. Kinesin-1 inhibition that caused a 6-fold decrease in anterograde flux caused a severe reduction in retrograde flux ($Khe^6/null = 14$ fold decrease), but decreases in specific retrograde run parameters were insignificant. In contrast, a kinesin-3 inhibition that caused a 5-fold inhibition in anterograde flux caused a relatively mild reduction in retrograde flux ($Unc-104^{O3.1}/null = 3$-fold decrease), and caused substantial defects in specific retrograde run parameters (Table 1, Barkus et al., 2008). So in the case of dynein-driven retrograde DCV transport, the influences
of the two kinesins are markedly different. This does not support a simple biophysical strain mechanism for activation of dynein by any opposing anterograde DCV motor. The contrast with the peroxisome results (Ally et al., 2009) could be due to different motor activation mechanisms for the different organelles, or to the different approaches used. Tests of in vivo domain swaps between Khc and Unc-104 could produce interesting new insights.

2.4.2 A requirement for two DCV kinesins

Regarding the issue of why inhibition of either kinesin-1 or kinesin-3 causes substantial decreases in anterograde run velocity (20-40%) and run length (2-3-fold), it is worth considering an unusual feature of kinesin-3 motor structure and function. Initial biochemical analysis of the Unc-104 homolog Kif1A suggested that it could function as a monomeric motor (Nangaku et al., 1994). Subsequent single molecule tests showed that Unc-104 monomers are not capable of fast processive motion unless artificially dimerized (Tomishige et al., 2002). Tests with liposomes showed further that Unc-104 clustering in lipid rafts facilitates fast processive movement (Klopfenstein et al., 2002). These results suggest that axonal DCVs carried solely by kinesin-3 will have difficulty sustaining long runs if motor copy number is low or if clustering is not robust. Thus, strongly dimerized kinesin-1 motors on DCVs could be important for bridging gaps in processive kinesin-3 activity.
Another reason for employing two anterograde motor types on a single vesicle could be to function along different parts of the transport path. A precedent for that sort of sequential transport with two different kinesin-2 motors has been found in sensory cilia of *C. elegans* neurons. Osm-3 and kinesin-II both function on a single intraflagellar transport (IFT) particle for anterograde movement along proximal axoneme doublet microtubules. Then Osm-3 alone continues IFT along distal singlet microtubules (Snow et al., 2004; Ou et al., 2005; Pan et al., 2006). It is evident that Unc-104 inhibition causes vesicle accumulation in neuronal cell bodies, but not in axons (Hall and Hedgecock, 1991; Pack-Chung et al., 2007; Barkus et al., 2008; Zala et al., 2013) while *Khc* mutations cause aberrant vesicle accumulation in axons (Figure 1 and Hurd and Saxton, 1996; Pilling et al., 2006), but not in cell bodies (our unpublished observations), suggesting that Unc-104 and Khc make different contributions along different parts of the transport path.

Post-translational microtubule modification could provide a molecular basis for this. Kinesin-1 preferentially transports vesicles along stable, acetylated microtubules as opposed to more dynamic, non-acetylated microtubules, while kinesin-3 functions well on both (Cai et al., 2009). Newly formed, dynamic microtubules predominate proximal to the cell body, while older, more stable microtubules predominate in the main axon (Brown et al., 1992). Thus kinesin-3 may be especially important for the initial movement of DCVs along dynamic microtubules from the cell body into the main axon. Then, both kinesin-1 and -3
could function cooperatively to drive fast processive transport along the main axon toward the terminal.
CHAPTER 3 AUTOPHAGY AND AXONAL SWELLINGS

3.1 INTRODUCTION

Axonal degeneration is a contributing factor to many neurodegenerative diseases. One of the early signs of neuronal stress is the appearance along axons of discrete swellings, also known as focal accumulations or spheroids. These axonal swellings accompany neurodegenerative diseases such as Alzheimer’s (Ohgami et al., 1992), lysosomal storage disorders (Walkley et al., 1991), infantile neuroaxonal dystrophy (INAD) (Yagishita, 1978) and motor neuron disorders such as amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease (CMT), and spastic paraplegia (Carpenter, 1968; Toyoshima et al., 1998; Kasher et al., 2009). In addition, axonal swellings have been observed accompanying other pathological conditions such as tumors, vascular lesions and demyelination-related disorders suggesting that they are common in neurons under duress and precede neuronal death (Yagishita, 1978). Despite the prevalence of axonal swellings, their biogenesis and how they influence neurodegeneration remains unknown.

Studies of axons have provided some insights into the composition of axonal swellings. Post-mortem analyses of tissues from patients with neurodegenerative diseases have shown that swellings are heterogeneous both in size and cytoplasmic contents. Electron micrographs revealed that they frequently contain all or a
combination of: interconnected membrane tubules, glycogen granules, abnormal neurofilaments, vesicles, mitochondria, multivesicular bodies and other darkly stained prelysosomal complexes (Yagishita, 1978). Because axonal swellings contain components carried by axonal transport, they were suspected to be secondary to axonal transport defects (Yagishita and Kimura, 1975).

Indeed, mutations in some genes encoding proteins essential for axonal transport have been shown to cause axonal swellings. Studies in Drosophila identified axonal swelling phenotypes in both kinesin-1 and dynein mutants (Figure 3-1) (Gepner et al., 1996; Hurd and Saxton, 1996; Martin et al., 1999). In addition, mutations or RNAi for genes that encode Klc, Milton, Aplip1, Unc76 and Roadblock, proteins that interact with kinesin-1 or dynein, can also cause swellings (Bowman et al., 1999; Martin et al., 1999; Gindhart et al., 2003; Horiuchi et al., 2005). The swellings in kinesin-1 and dynein mutants have similar contents: mitochondria, small vesicles, membrane tubules, large multivesicular bodies (MVBs), and darkly stained vacuoles. The morphologies of the vacuoles and MVBs suggest that they are part of the autophagosome-lysosome degradation pathway. Mutations in the Kif5A gene, which encodes the anterograde kinesin-1 motor, have been found to cause some forms of spastic paraplegia (Reid et al., 2002) and CMT (Goizet et al., 2009; Crimella et al., 2011). Mutations of the retrograde motor dynein have been found to cause some cases of ALS (Munch et al., 2004) and SBMA (Puls et al., 2005). These studies of humans confirm that when axonal transport machinery is disrupted, discrete
swellings form along axons filled with organelles, including MVBs and dark vacuoles that suggest increased autophagy activity.
Figure 3-1 Mutations in kinesin-1 and dynein cause swellings in axons. Transmission electron micrographs of segmental nerve cross sections from (A) wild-type, (B) kinesin-1 mutant, (C) wild-type, and (D) dynein mutant larvae. In A and B, arrowheads mark the plasma membranes of single axons. In C and D, normal axons are marked by arrows while swollen axons are marked by arrowheads. Scale bars=500nm. G, glial cells; gn, peripheral glial cell nucleus; M, mitochondria; p or PLV, prelysosomal vacuoles; MV, multivesicular body. A and B are images from (Hurd and Saxton, 1996). C and D are images from (Martin et al., 1999).
Autophagy is a pathway for the breakdown of organelles and macromolecules. It has important housekeeping functions within the cell by constitutively removing damaged organelles and larger macromolecular complexes such as protein aggregates (Deter et al., 1967; Jaeger and Wyss-Coray, 2009; Yang and Klionsky, 2010; Nixon, 2013). Increased autophagy activity can be triggered by metabolic stresses, including nutrient deprivation (Seglen and Gordon, 1982) and hypoxia (Figure 3-3) (review in (Nixon, 2013)). The mechanism of autophagy is complex with molecular details that are not yet well understood. In general, it starts with the initiation of an isolation membrane (phagophore) near the cytoplasm targeted for degradation (degradation substrates), perhaps by the fusion of vesicles (Suzuki et al., 2001). The phagophore membrane expands, growing around and enclosing the degradation substrates in a double membrane autophagosome. The autophagosome then may fuse with endosomes to form multivesicular bodies called amphisomes (Tooze et al., 1990; Punnonen et al., 1993; Eskelinen, 2005). Finally, fusion of amphisomes with lysosomes initiates degradation (Figure 3-2) (reviewed by (Mizushima, 2007)).

Autophagy induction is negatively regulated by TOR (Target of Rapamycin), so inhibition of TOR increases autophagy and activation of TOR inhibits autophagy (Figure 3-3) (Blommaart et al., 1995; Noda and Ohsumi, 1998). The mTOR inhibitor, rapamycin can reduce neurodegeneration in various transgenic mouse disease models including Huntington’s, Alzheimer’s, prion, and Parkinson’s diseases, and spinocerebellar ataxia type 3 (Ravikumar et al., 2002; Ravikumar et al., 2004; Menzies et al., 2010; Spilman et al., 2010). It is believed that autophagy promotes the
clearance of the protein aggregates common to these diseases and thus has beneficial
effects on diseased neurons. This interpretation is clouded by questions about
whether the aggregates are toxic or are actually protective (Nixon, 2013). However,
the fact remains that TOR inhibition by rapamycin has a protective effect in some
models of neurodegenerative disease.

The rapamycin results, along with the abundance of MVBs and other
autophagosomal organelles in axonal swellings and the fact that axonal transport
defects cause swellings and axon degeneration led me to the following hypothesis
about the relationships between transport, swellings, autophagy, and degeneration: 1) poor transport causes aberrant delivery/retrieval dynamics of organelles resulting in
their abnormal distribution in axons, 2) the abnormal distribution causes aberrant
physiology that results in axonal swellings and causes degeneration; and 3) autophagy is activated to suppress swellings by clearing mislocalized organelles, and
to thus suppress axon degeneration. To test these ideas, undergrad colleagues and I
used various approaches to inhibit or overactivate autophagy pathway proteins in
Drosophila motor neurons that had swellings caused by overexpression of
JIP1/Aplip1, a kinesin-1 interacting protein. Our results show that treatments that
should activate autophagy (inhibition of TOR or overexpression of Atg1) decrease
JIP1/Aplip1 induced axonal swellings, while treatment that should suppress
autophagy (TOR up-regulation) increased axonal swellings. These results suggest
that increasing autophagy can remove axonal swellings.
Figure 3-2 Macroautophagy in mammalian cells. An isolation membrane forms around a portion of the cytoplasm or organelles targeted for degradation. The completed double membrane autophagosome can fuse with other endosomes. Eventually, fusion with lysosomes causes acidification and degradation of the autophagosome contents. Image adapted from (Mizushima, 2007).
Figure 3-3 TOR signaling pathway in mammals. 
There are two main TOR signaling pathways -- The rapamycin sensitive mTORC1 (Mammalian TOR Complex 1) controls downstream events that determine cell size. Rapamycin insensitive mTORC2 (Mammalian TOR Complex 2) is involved in actin organization which determines cell shape. mTORC1 responds to growth factors, stress and nutrients. It is unclear whether mTORC2 responds to the same factors as mTORC1. Arrows represent activation, bars represent inhibition. Figure from (Wullschleger et al., 2006).
3.2 MATERIALS AND METHODS

Fly Stocks

Drosophila were cultured with 12hr light-dark cycles at 22-25°C, according to standard protocols (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). Fly lines carrying UAS-transgenes with small hairpin RNAs for RNAi whose expression can be induced by Gal4 were obtained from the Transgenic RNAi Project (TriP): P{TRiP.HMS00904}attP2 (TOR), P{TRiP.HMS01114}attP2 (TOR), P{TRiP.GL00047}attP2 (Atg1), P{TRiP.HMS01244}attP2 (Atg5), P{TRiP.HMS01358}attP2 (Atg7), P{TRiP.HMS01328}attP2 (Atg8a), P{TRiP.HMS01245}attP2 (Atg8b), P{TRiP.HMS01246}attP2 (Atg9), P{TRiP.HMS01153}attP2 (Atg12), P{TRiP.HMS01193}attP2 (Atg18). Other fly strains used were UAS-Rheb, genotype w[ ]; P{w[+mC]=UAS-Rheb.Pa}3. The UAS-Atg1 line, yw; UAS Atg1[6B] was a gift from Thomas Neufeld.

Previous studies showed that expression of P{UAS-Aplip1.FLAG} using the D42 motor neuron driver P{GawB}D42, causes larval tail flipping behavior, 100% lethality by the pupa stage, and axonal swellings that contain vesicle proteins and Aplip1 (Horiuchi et al., 2005). In my work, axonal swellings were imaged in neurons of live larvae using a neuropeptide-GFP fusion protein (ANF::GFP) that is targeted to the lumens of DCV (Rao et al., 2001). P{w[+mC] UAS-ANFGFP}3 was expressed in
motor neurons using the neuron specific D42 driver. To image autophagosomes, $P\{UASp-mCherry-Atg8a\}$ was recombined with the motor neuron driver $P\{GawB\}VGlut^{Ok371}$ (Ok371) on chromosome 2. Except where noted, all stocks were obtained from the Bloomington Drosophila stock center.

**RNAi Screen**

Standard meiotic recombination was used to generate a chromosome 3 carrying the D42 motor neuron driver, the $P\{w^{+mc} \ UAS-ANFGFP\}3$ responder and $P\{UAS-Aplip1.FLAG\}$, which causes axonal swellings (Horiuchi et al., 2005). The recombinant chromosome, which will be referred to as $D42, Aplip1, ANFGFP$ was balanced over $TM6B, P\{tubP-GAL80\}OV3,Tb^l$. We tested each candidate UAS-RNAi by crossing its fly stock to the $D42, Aplip1, ANFGFP$ / $TM6B, P\{tubP-GAL80\}OV3,Tb^l$ stock. The non-tubby larvae carried both a candidate RNAi and $D42, Aplip1, ANFGFP$. The overexpression of Aplip1 specifically in motor neurons causes axonal swellings that contained ANF::GFP. The non-tubby larvae were imaged by time-lapse spinning disc confocal microscopy to assess levels of axonal swellings in their segmental nerves.

**Live Imaging**

Imaging was done with a Nikon Eclipse TE2000-E Inverted Spinning Disk Laser Confocal Fluorescence Microscope. The nervous systems of third instar larvae were viewed by placing live larvae ventral side down in 50ul of halocarbon oil. A
cover slip, spaced by two layers of clear packing tape on each edge was placed over the larvae and partially sealed with wax. An anesthetic, desflurane (Baxter), was wicked underneath the cover slip and then the wax seal was finished. Images of nerves in abdominal segments were recorded using a 10x (NA 0.3) objective lens in the anterior, middle, and posterior regions. Exposure time for each image was 500ms. The laser and other settings of the microscope system were kept consistent through all imaging sessions.

*Imaging Dissected and Fixed Neuromuscular Systems*

DCVs filled with ANF::GFP were imaged in 3rd instar larvae neuromuscular preparations that were dissected in standard phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS for 10 minutes. Fixed neuromuscular preps were mounted on glass slides with 50ul of VectaShield (Vector Laboratories), covered with a glass coverslip, and sealed with nail polish. Images were recorded using the spinning disk confocal microscope using a 40X (NA 0.75) dry lens. Image exposure time was 500ms. The laser and other microscope system settings were kept consistent during all imaging sessions.
3.3 RESULTS

3.3.1 Autophagic membranes are localized in focal accumulations in axons

If the large osmiophilic (darkly stained) vacuoles seen to dominate the centers of axonal swellings by transmission electron microscopy (Hurd and Saxton, 1996; Martin et al., 1999) are part of the autophagy pathway, we should see an accumulation of autophagic proteins in swellings. Atg8 is a transmembrane protein involved in membrane expansion during autophagy and is a standard marker for autophagosomes (Mizushima, 2004). To determine if the axonal swellings contain autophagosomes, \( P\{UAS-Aplip1.FLAG\} \), \( UAS-ANFGFP \) and \( UASp-mCherry-Atg8a \) were coexpressed in \textit{Drosophila} motor neurons using the \textit{D42} and \textit{Ok371} motor neuron drivers. In control animals that did not carry the \textit{Aplip1} transgene, ANF::GFP was uniformly distributed along nerves. mCherry-Atg8 fluorescence appeared somewhat punctate in cell bodies of the ventral ganglion but was mostly diffuse in axons. In animals that overexpressed Aplip1, axonal swellings filled with ANF::GFP were abundant along the nerves and mCherry-Atg8 punctae were present in the ventral ganglion, in the nerves, and were abundant in axonal swellings (Figure 3-4). This suggests heightened autophagy activity in neurons that suffer from transport defects, especially in axonal swellings.
Figure 3-4 mCherry-Atg8 is detected in axonal swellings.
A) Nerve from a control animal expressing ANF::GFP and the autophagosome marker, mCherry-Atg8 (red) is shown. B) In animals that coexpress Aplip1, axonal swellings of ANF::GFP are visible throughout the nerve. mCherry-Atg8 is commonly localized in these ANF::GFP swellings. Scale bar=5µm
Figure 3-5 Design of RNAi screen to identify modifiers of axonal swellings. Standard meiotic recombination was used to put the \textit{UAS-Aplip1}, \textit{UAS-ANFGFP} transgenes together with the motor neuron D42 GAL4 driver on the same chromosome. This stock was crossed to different candidate \textit{UAS-RNAi} lines. The progeny (non-tubby larvae, shown in box) that carried the \textit{Aplip1} and \textit{UAS-RNAi} transgenes were then imaged live for changes in axonal swellings compared to larvae without \textit{UAS-RNAi} expression.
3.3.2 TOR inhibition reduces axonal swellings

The concentration of autophagosomes in axonal swellings is consistent with our hypothesis that autophagy has a role in suppressing swellings. To test this, we used neuron-specific RNAi to inhibit the expression of proteins in the autophagy pathway and then imaged axons in live larvae. Nerves from larvae that carried Aplip1, ANFGFP and a candidate RNAi were compared to controls, which did not carry the RNAi Figure 3-5. If autophagy contributes to the clearance of axonal swellings, inhibition of autophagy by RNAi should lead to more swellings. If autophagy has no functional role in axonal swellings, RNAi of autophagy (Atg) proteins should have little influence on axonal swellings.

We tested the influences of 9 different proteins that function in the autophagy pathway (Figure 3-6). Starting with TOR, whose inhibition activates autophagy (Noda and Ohsumi, 1998; Ravikumar et al., 2004), we studied two different TOR RNAi constructs. Both caused a reduction in the size and number of Aplip1-induced axonal swellings. To check this with higher quality images, we dissected and fixed 3rd instar larvae and imaged the exposed nerves (Figure 3-7). Consistent with the live animal tests, there was a clear decrease in the amount and size of axonal swellings in TOR RNAi nerves.
Figure 3-6  A decrease in axonal swellings in living TOR RNAi larvae. ANF::GFP expression in neurons (D42 GAL-driver) was used to reveal axonal swellings. Nerves of live 3rd instar larvae over expressing Aplip1 plus one candidate RNAi were imaged in the anterior (A, D, G, J), middle (B, E, H, K), and posterior (C, F, I, L) regions. Representative images from the different RNAi genotypes are shown. (A-C) Aplip1 controls (Aplip1 OE). (D-F) TOR RNAi larvae showed a marked reduction in the number of axonal swellings (arrows). (G-I) Atg7 or (J-L) Atg12 RNAi did not cause a similar reduction in the number of swellings. V = ventral ganglion, sg = salivary glands. These images are representative of 3 live animals examined for each genotype. Scale bar = 100µm.
Figure 3-7  *TOR* RNAi effects seen in dissected and fixed larval neuromuscular systems.

ANF::GFP expression in neurons (D42 GAL-driver) was used to reveal axonal swellings. 3rd instar larvae were dissected to optimize imaging of their nerves. Nerves were imaged in the anterior (A, D, G), middle (B, E, H), and the posterior (C, F, I) regions of larvae. (A-C) Images from larvae overexpressing Aplip1 (Aplip1 OE). (D-F) Images from a larva overexpressing Aplip1 plus *TOR* RNAi show a reduced number of swellings. (G-I) Images from larvae expressing *TOR* RNAi without APLIP1 overexpression. The small GFP foci in (G, H, I) is also seen in wild type animal when ANF::GFP is expressed with the D42 driver and is likely not an effect of the RNAi. These images are representative from the following sample sizes: Aplip1 OE, n=15, Aplip1 OE+*TOR* RNAi n=7, *TOR* RNAi, n=10. Scale bar = 20µm
3.3.3 TOR activation increases axonal swellings

If inhibition of TOR expression reduces swellings, over activity of TOR might cause an increase in swellings. Previous studies have shown that TOR overexpression has a dominant negative effect, resulting in phenotypes similar to TOR inhibition (Hennig and Neufeld, 2002), so a direct approach will not work. To circumvent this, we overexpressed Rheb, an upstream activator of TOR (Hennig and Neufeld, 2002). When Rheb and Aplip1, ANFGFP were coexpressed in larval motor neurons, there was a clear increase in the number and size of axonal swellings, compared to control animals (Figure 3-8). Along with the TOR inhibition tests, this suggests that TOR activity facilitates the formation of or inhibits the removal of axonal swellings caused by Aplip1 overexpression.

3.3.4 Atg1 overexpression suppresses axonal swellings

TOR controls important cellular processes other than autophagy, including: protein synthesis, ribosome biogenesis and mRNA transcription (Wullschleger et al., 2006). This raised the question of whether or not the TOR influences on swellings that we observed were specific to its activity in the autophagy pathway. To address this, we tested Atg1, a downstream target of TOR and an early component of autophagy activation. Loss of Atg1 inhibits autophagy while overexpression of Atg1 is sufficient to activate autophagy, independent of TOR (Scott et al., 2004).
overexpress Atg1, *UAS-Atg1* was induced in *Drosophila* motor neurons along with *Aplip1*, using the OK371 Gal4 driver. Axonal swellings were reduced, compared to control animals (Figure 3-9). This effect was similar to that of TOR inhibition. These findings, along with the TOR results described above, support a role for autophagy in suppressing axonal swellings.
Figure 3-8 Rheb overexpression increases the number and size of axonal swellings in larvae over expressing Aplip1. ANF::GFP expression in neurons (D42 GAL-driver) was used to reveal axonal swellings. 3rd instar larvae were dissected, fixed and their nerves were imaged in the anterior (A,D,G), middle (B,E,H) and the posterior (C,F,I) regions of the animals. (A-C) show images from a larva overexpressing Aplip1 (Aplip1 OE). (D-F) show images from a larva overexpressing both Aplip1 and Rheb (Aplip1 OE+Rheb OE). (G-I) show images from a larva overexpressing Rheb (Rheb OE). These images are representative of the average results from the following sample sizes: Aplip1 OE, n= 9, Aplip1 OE+Rheb OE n= 15, Rheb OE, n=7. Scale bar = 20µm.
Figure 3-9 Atg1 overexpression decreases the size and number of axonal swellings in larvae overexpressing Aplip1.

ANF::GFP expression in neurons (D42 GAL-driver) was used to reveal axonal swellings. 3rd instar larvae were dissected, fixed and their nerves were imaged in the anterior (A,D), middle (B, E), and posterior (C, F) region of the animal. (A-C) are images from a larva overexpressing Aplip1 (Aplip1 OE). (D-F) are images from a larva overexpressing both Aplip1 and Atg1 (Aplip1 OE+Atg1 OE). These images are representative of the average results from the following sample sizes: Aplip1 OE, n= 6. Aplip1 OE+Atg1 OE, n= 15. Comparison with Atg1 expression alone was not done, because the animals died before developing to the third instar larval stage. Scale bar = 20µm.
3.4 DISCUSSION AND FUTURE EXPERIMENTS

Axonal swellings have long been observed to be associated with degenerating or injured neurons. However, the cell biology of swellings and their functional relationship to neurodegeneration remain unknown. The axonal swellings of kinesin-1 and dynein mutants are filled with structures resembling components of the lysosomal-degradative pathway (Figure 3-1). Using mCherry-Atg8, we saw accumulations of the autophagy-lysosomal pathway marker in axonal swellings marked by ANFGFP. In addition, two different approaches to activation of autophagy, TOR inhibition or Atg1 overexpression, suppressed swellings, and inhibition of TOR by Rheb overexpression increased swellings. These results suggest that autophagy either inhibits the biogenesis of or facilitates the clearance of axonal swellings.

If this interpretation is accurate, swellings from axonal transport defects caused by inhibition of any component of the transport machinery (e.g.: Khc, Klc, Milton, and Dhc) should also be reduced by autophagy activation. My preliminary studies of swellings in animals have generated mixed results. TOR RNAi did suppress Khc RNAi swellings, however Atg1 overexpression did not. More tests need to be done, but if these results are accurate they suggest differences between swellings caused by Aplip1 overexpression and Khc inhibition. One interesting explanation could be that the primary functions of Khc and Aplip1 are different.
Khc is the kinesin-1 force generator that influences virtually all axonal transport cargoes, while Aplip1 is a linker between Khc and a subset of its axonal cargoes. Therefore, the effects of TOR and Atg1 on axonal swelling may be different between swellings caused by a defective motor and those caused by a defective cargo linker. Future work will test the influences of TOR and Atg1 on axonal swellings caused by inhibition of Aplip1, as well as by inhibition of Klc, Milton, and Dhc.

The question of whether the removal of axonal swellings via autophagy can prevent the lethality commonly associated with mutations that cause swellings remains open. Recent studies from our lab showed that when kinesin-1 mutants were grown in nutrient restricted conditions, there was an increase in the number of larvae surviving to the pupal stage (Djagaeva et al., 2013). Since autophagy can be induced by nutrient-poor conditions (Seglen and Gordon, 1982; Klionsky and Emr, 2000; Chang et al., 2009), the increase in kinesn-1 mutant survival could be a result of autophagy-induced suppression of axon swellings and degeneration. However, my preliminary tests suggest that axonal swellings in kinesin-1 mutants are not reduced by nutrient restriction (data not shown). Thus, the increased survival of kinesin-1 mutants under nutrient restriction may be due to changes in physiology that are not influenced by axon degeneration and autophagy.

Interestingly, in our Atg1 studies, we found that expression of Atg1 in motor neurons in a background without Aplip1 overexpression causes severe development
defects. Animals overexpressing Atg1 in motor neurons showed severely reduced growth rates and most died by the 3rd instar larval stage. This is consistent with a previous report that showed that cells with Atg1 overexpression were TUNEL positive and displayed caspase activation, suggesting that Atg1 can promote cell death (Scott et al., 2007). Why does co-overexpression of Aplip1 suppress these Atg1 phenotypes?

Collectively, my preliminary data suggest that axonal swellings, which are associated with neurodegeneration, can be reduced by an increase in autophagy. Clearly, the causal relationship of swellings and neurodegeneration requires further study. Future work will focus on the effects of TOR RNAi and Atg1 expression on the survival of Aplip1 defective mutants (Aplip1Ek4) and with the UAS-Aplip1 transgene). Another focus will be on addressing the effects of TOR and Atg1 on axonal swellings – does autophagy prevent the formation of axonal swellings or accelerate the removal of swellings? Lastly, is the relationship between Atg1 and Aplip1 via autophagy? By answering these questions, we can get a better understanding of the relationship of axonal swellings, autophagy, and axon degeneration in diseased neurons.
SUPPLEMENTAL FILES

Available at http://tinyurl.com/saxtonlab-angelinelim-sup01

All movies are oriented such that the ventral ganglion is located to the left; therefore, DCV or mitochondria movement from left to right represents anterograde transport and movement from right to left represents retrograde transport. The D42 motor neuron driver controls expression of the UAS-ANF::GFP responder in Movie 1 and 2. The Ok371 motor neuron driver controls expression of the UAS responders in Movies 3 to 8.

Movie 1

Axonal transport of ANF::GFP labeled DCVs in segmental nerve of a control larva. (playback at 5x realtime)

Movie 2

Axonal transport of ANF::GFP labeled DCVs in a segmental nerve of a Khc mutant larva (Khc0/Khc27). (playback at 5x realtime)

Movie 3

Mitochondria labeled with GFP shows normal transport in a segmental nerve of a control larva. (playback at 10x realtime)

Movie 4

Mitochondria labeled with GFP are mostly absent in the motor neurons of larvae expressing Milton RNAi. The few mitochondria imaged are mostly stationary. (playback at 10x realtime)
**Movie 5**

Axonal transport of ANF::GFP labeled DCVs in a segmental nerve of a control larva. (playback at 5x realtime)

**Movie 6**

Axonal transport of ANF::GFP labeled DCVs in a segmental nerve of a larva expressing Milton RNAi in motor neurons. Note that the movement of DCVs in the Milton RNAi larval nerve is very similar to those in the control larva in Movie 5. (playback at 5x realtime)

**Movie 7**

Axonal transport of ANF::GFP labeled DCVs in a segmental nerve of a control larva. (playback at 5x realtime)

**Movie 8**

ANF::GFP transport of ANF::GFP labeled DCVs in segmental nerve of a Dhc mutant larva. (playback at 5x realtime)
REFERENCES


