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EXAMINING RIBOSOMAL LOCALIZATION BASED ON MICROTUBULE POLARITY IN *DROSOPHILA MELANOGASTER* NEURONS

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ABSTRACT

Ribosomes are crucial to cellular function and development, as they are responsible for translation of mRNA to produce proteins that are vital to life; this holds true in neurons because they must be preserved for an organism's entire life. Understanding ribosomal localization in neurons can help build the framework to answer key questions about regeneration and disease, especially when comparing axons and dendrites. Interestingly, the localization is differentiated based on the neurite extending from the cell body in a plethora of organisms; ribosomes accumulate in the soma and dendritic branch points, but not the long axon, of *Drosophila melanogaster* dendritic arbor (da) neurons. This body of work aims to uncover how exactly ribosomes are being trafficked to dendritic branch points. Different approaches were utilized to do this, including the knockdown of a motor protein and the disruption of normal microtubule polarity to yield scenarios differential to the uniform polarity of the *Drosophila* neuron. I uncovered that the knockdown of Dynein, which is the minus-end-out motor protein, significantly decreased ribosomal localization in the dendritic branch points. Furthermore, uniform minus-end-out microtubule polarity in the dendrites was altered by both knocking down a microtubule stabilizing protein and inducing the change to plus-end-out polarity via axon injury to determine if this affected ribosomal localization. In both such cases, it was determined that ribosomal trafficking was decreased in branch points that had increased plus-end-out character. Each experiment supports the hypothesis that ribosomal trafficking is dependent on minus-end-out microtubule polarity. This can lead to insight on differential means of regeneration and upholding between axons and dendrites, as well as help explain the localization of different organelles and crucial proteins that behave in the same manner.

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Chapter 1

Introduction

Neurons across organisms

Both the nervous system and its function remain as one of the most fascinating components of the human body. Neurons are the major cell of this system, responsible for a plethora of tasks. These highly specified cells play key roles in not only humans, but organisms ranging from fish to insects, too. The neuron exhibits a basic structure and role (Figure 1). The cell body, or soma, is home to the nucleus and a majority of the neuron's organelles. Cellular signals, both chemical and electrical, are received and transmitted through the soma via the dendrites, which consists of a highly branched arbor. The dendrites are responsible for receiving and carrying on signals from the axon. The axon is a long, whip-like protrusion on the opposite end of the soma; this meets with the subsequent dendritic arbor at the synapse. Axons, in turn, conduct the action potential across the cell membrane, which is carried outward to the axon terminus and yields a response[1].

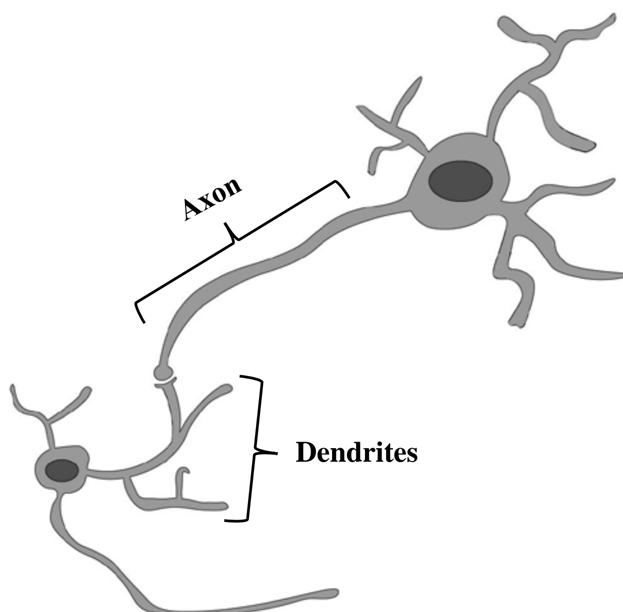


Figure 1. Basic structure of neuron. A visualization of the structure of the neuron and its different cellular protrusions. The long axon of the cell transmits electrochemical signals to the adjacent neuron at the synapse. The dendrite is a highly branched region that receives cellular signals from other neurons.

When problems arise and neurons are deteriorated, the integrity of the nervous system can be compromised. Neuronal cell death is seen in a plethora of neurodegenerative diseases and trauma, often resulting from unknown causes and mechanisms[2]. Neurons cannot divide once differentiated and can only be derived from a subgroup of predetermined neural stem cells. This neurogenesis, or production of neurons, ceases towards the later stage of life; hence, it is of utmost importance to maintain these crucial cells throughout the life of an organism. Thus, studying the development and state of these cells can lead to a better understanding of how to prevent and ultimately treat neurodegenerative disease and degeneration as a whole.

Microtubule polarity in neurons

When understanding neuronal function and structure, it is important to regard the major framework of neurons: the microtubules. These are a distinct structure present in all cells, keeping them stable and playing key roles in intracellular trafficking. The microtubule is a unique polymer, constructed of alpha and beta tubulin dimers. These are polymerized from the gamma-tubulin ring complex (ϒ-TURC), which possess many proteins for nucleation like gamma tubulin[3]. The growing end of this polymer is denoted as the “plus-end” of this structure, and can be visualized by fluorescently tagged end binding protein 1 (EB1). The opposite end is labeled as the “minus-end” of the microtubule. Other proteins are associated with the microtubule structure. These include microtubule associated proteins (MAPs) that are responsible for crosslinking these structures, and specific end binding proteins that act to stabilize microtubules from depolymerization[4].

Perhaps the most interesting proteins that interact with microtubules are the motor proteins, which hold great value in many biological functions. Kinesin and Dynein are the microtubule-associated cytoskeleton motor proteins. These are extremely important in neurons and play roles in neuron polarization, neurotransmission, neurite extension, and cellular shape. Dysfunction of these two key motors often lead to disease in humans[5]. Due to the long protrusions in neurons, microtubules and motor proteins are extremely important. Certain proteins and organelles must be trafficked extremely far distances from the soma in anterograde transport, or from distal neurites back to the soma via retrograde transport; microtubules make this possible[1]. This is crucial to the neuron, as an axon can extend to distances greater than three orders of magnitude of the cell body[6].

Microtubules can be regarded as "train tracks," and they are extremely important in carrying organelles and specific proteins to and from the soma, out to the different neurites. The trafficking of certain organelles and specific proteins often happens in a differential manner, as certain cellular components localize to the dendrites, but not the axons, and vice versa. Components that specifically localize to the dendrites include the rough endoplasmic reticulum[7], the Golgi complex, ribosomes, and GABA receptors; this is countered by synaptic vesicles[8] and neurofilaments[9] in the axon.

Perhaps the simplest way to explain this phenomenon is through neuronal microtubule polarity. While diffusion can only traffic certain cellular components a short distance from the soma, specific trafficking to farther distances can be facilitated by microtubules. This occurs through individual motor proteins binding cargo and traveling in a walking fashion along the microtubule. Each motor protein moves in a specific direction. For example, kinesin travels in plus-end direction, while dynein travels in the minus-end direction[10]. The differential movements of these proteins are extremely beneficial in the cell, especially in neurons.

Interestingly, the axon and dendrites have opposite microtubule polarity in *Drosophila* neurons; while each separate protrusion carries a different purpose, they also exhibit opposite microtubule polarities. The many branches of dendrites exhibit minus-end-out microtubule polarity, while the opposing axon is plus-end-out[8]. This is a uniform polarity (Figure 2):

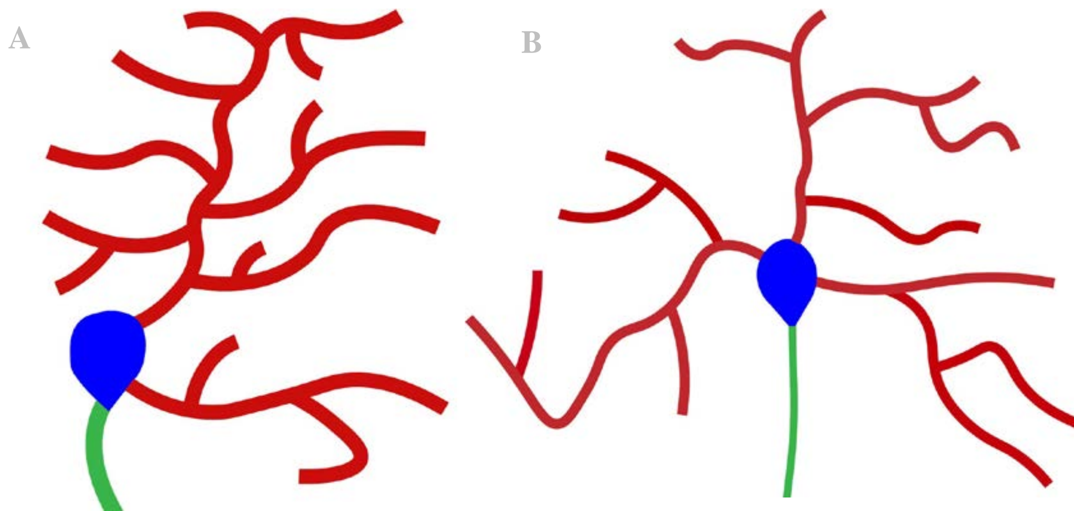


Figure 2. Uniform microtubule polarities of neuronal sections. Diagram denoting microtubule polarity in *Drosophila* neurons. Class I ddaE (A) and Class IV ddaC (B) neurons hold separate function and overall structure, but maintain the same microtubule polarity in the axon and dendrites. Minus-end-out microtubule polarity is colored red, while plus-end-out microtubule polarity is colored green.

This is not uniform across all organisms, however. The dendrites of both rat hippocampal neurons[11] and frog brain cells[12] have been established to exhibit mixed microtubule polarity. The uniform nature of plus-end-out microtubules in the axon and minus-end-out microtubules in the dendrite make the *Drosophila* a powerful tool for examining trafficking and the implications this has on neurons.

There are two separate *Drosophila* neuron classes that we use for the examination of these biological questions. The class I ddaE, and class IV ddaC neurons are dendritic arborization (da) neurons[13]. While these are both sensory neurons existing in the abdomen of the larva and possess many dendrites, they possess differences. These classes of neurons are morphologically distinct and hold different levels of complexity. Class I neurons are far less intricate and occupy a smaller area than their counterpart[14]. However, the uniform "comb" structure of class I ddaE neurons leaves an optimal structure for examining a multitude of

dendritic branch points. In each of the da neurons, the multiple dendrites make these neuron subclasses an ideal model of study.

Importance of ribosomes and localization

Ribosomes are crucial to cellular function and development, as they are responsible for translation of mRNA to produce proteins that are vital to life. In polarized cells, like neurons, ribosomes are critical for the specific local environments of the cell and localized protein synthesis[15]. While the ribosome has been a topic of interest in molecular biology studies, the trafficking of ribosomes and protein biosynthesis process carried on by eukaryotic ribosomes is extremely complex and not yet fully understood[16]. Understanding this localization in neurons can help answer key questions about regeneration and disease, especially when comparing axons and dendrites. Interestingly, localization of ribosomes in *Drosophila* neurons, along with many other model organisms, is differentiated based on the neurite extending from the cell body.

In *Drosophila* neurons, ribosomes accumulate at dendritic branch points, but not axons[17] (Figure 3). This statement has been a debate amongst scientists for years, as certain studies have seen polyribosomes and local synthesis of important proteins in the axon[18]. Ribosomal localization differs across a plethora of organisms, however. For example, fluorescently-labeled ribosomes have been determined to accumulate in the axons and synaptic terminals of *Caenorhabditis elegans* neurons[15]. Ribosomal RNA antibodies have show ribosomes to localize to axons in mammalian peripheral neurons, too[19]. These differences in trafficking across organisms can be due to the difference in axonal microtubule polarity.

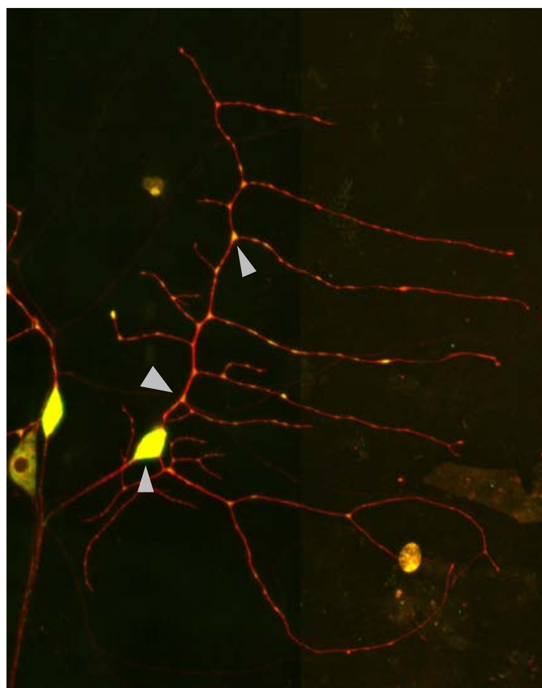


Figure 3. Ribosomal localization in *Drosophila* neuron. Ribosomes, visualized by tagging the ribosomal protein L-10 with YFP, are seen to localize to the soma and dendritic branches of class I ddaE neurons.

While the trafficking of many neuronal components have been identified and understood, the process in which ribosomes are moved out from the soma remains a major question. This project aims to answer the question of ribosomal trafficking in *Drosophila* neurons.

Hundreds of dendritic mRNAs have been uncovered in hippocampal neurons, including important mRNAs encoding cytosolic, cytoskeletal, and integral membrane proteins. While these dendritic mRNA's have been extensively studied and determined to be trafficked in the form of large granules to their final destinations in dendrites[20], the primary method of ribosomal trafficking remains unknown. This is a significant question, as the products of translation in the dendrites include important proteins like neurotransmitter receptors, scaffolding proteins and signal transducing enzymes [21]. Complications occur when these proteins are not synthesized and

could potentially be drawn back to ribosomes not reaching the dendrites. Localization of ribosomes and translation in dendrites are of major importance, leading to abnormal neuronal function and fragile X mental retardation (FXMR) when compromised[22]. A greater understanding on neuronal biology and function is a necessity when digging deeper into human physiology and seeking cures to certain neurodegenerative diseases.

In addition, ribosomes have been identified in electron micrographs to be highly concentrated in the cell body and proximal dendrites of mature vertebrate neurons, with lesser localization in distal dendrites[23]. The differing local translation of proteins between axons and dendrites underlie several important cellular processes, including memory, cellular survival, and formation of synapses [24]. The diversity of microtubule polarity within neurons could explain why these organelles are only being transported to the dendrites. Studying ribosomes can lead to an understanding for the proteins and protein synthesis involved in dendrite regeneration. When regarding the differential localization of ribosomes in the neuron, and regarding the microtubule polarity of each neuronal section, a likely candidate for the trafficking of these ribosomes is Dynein, a motor protein transports cargo by "walking" on microtubules in the minus-end-out direction[10].

Studying Dynein transport can lead to hints on trafficking of other organelles that localize specifically to minus-end-out dendrites; this includes the rough endoplasmic reticulum (ER) and the Golgi complex, which show similar localization to ribosomes [23]. Thus, experimental results will give great insight to a mean of transportation in *Drosophila* neurons. Each outcome provides a greater understanding into intracellular trafficking and function. While the results of this experiment would lead to major implications on neuronal research, the range of impact can span much greater than what meets the eye.

Strategies to test ribosomal localization

Of all available model organisms, the *Drosophila melanogaster* remains as one of the most reliable and utilized tool in cellular biology. Better known as the common fruit fly, this organism leaves a perfect platform to examine this question, as well as many significant questions in developmental biology. Advantages of utilizing *Drosophila* include an extremely quick rate of reproduction, no true moral factors, and a diverse range of potential experiments. In addition, the ability to examine individual neurons and their specific regions is of great utilization in the field. Powerful systems have been developed, which allow the visualization of certain cellular components via fluorescent protein. Specifically, the method of fluorescently tagging the ribosomal protein L-10 has been proven to be successful in examining ribosomal localization[17].

I have hypothesized that ribosomal localization is dependent on minus-end-out microtubule polarity in *Drosophila* neurons. The major question of this work asks how ribosomes are differentially trafficked to dendrites, but not the axon. To address this, we used a combination of approaches to either alter uniform microtubule polarity or knockdown target proteins. In each experiment, fluorescent microscopy was utilized to examine ribosomal localization at dendritic branch points under different conditions.

Chapter 2

Results

YL-10 signal localizes to proximal axon, but is a viable marker for ribosomes

Before experimentation could be conducted regarding the major question, it was imperative to select an optimal ribosomal marker. The best way to visualize ribosomes in each experiment was to tag ribosomal protein L-10 with yellow fluorescent protein (YFP)[25]. Two fluorescent proteins, YL-10 A and YL-10 6.2, were utilized (Figure 4). Though containing different sequences and tagging different proteins of the ribosome, these were both sufficient in measuring ribosomal accumulations in separate experiments:

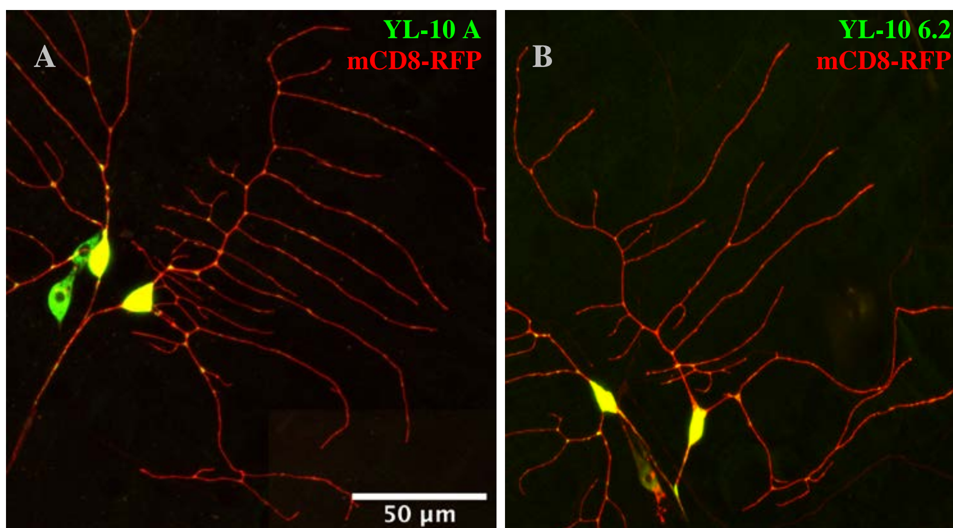


Figure 4. Comparing each ribosomal marker. The ribosomal markers utilized in the fluorescent microscopy experiments were YL-10 A (A) and YL-10 6.2 (B). Both fluorescent ribosomal markers were sufficient in tagging ribosomes for further quantification.

It is seen in these images that there is an accumulation of YFP in the proximal axon. This contradicts not only previously established principles on the nature of ribosomal localization[17], but also the hypothesis that minus-end-out microtubules localize ribosomes. However, this is most likely due to the local diffusion of these ribosomes. The axon initial segment (AIS) serves as a diffusion barrier, but is located some distance from the cell body on the proximal axon of ddaE neurons[26]. This can explain why ribosomal protein is localizing to the proximal axon. Several additional experiments were conducted to understand this axonal localization and validate the YL-10 marker.

First, a fluorescent recovery after photobleaching (FRAP) assay was conducted on these neurons tagged with YL-10. The purpose of this experiment was to confirm that this fluorescent marker was tagging the ribosomes and not another cellular component. Since ribosomes are rather large substituents of the cell, we hypothesized that the YFP signal would not recover after exposure to extreme bleaching. This was compared to a GFP-tagged ϒ-tubulin tester line as a control, which has been established to recover fluorescence roughly 40 seconds after photobleaching[27]. In addition, this fluorescent protein showed similar localization to the YL-10, so the experiment also served to confirm that gamma tubulin-GFP was not the marker being used. Images were captured in certain cellular regions showing fluorescence before, directly after, and 2.5 minutes after photobleaching (Figure 5):

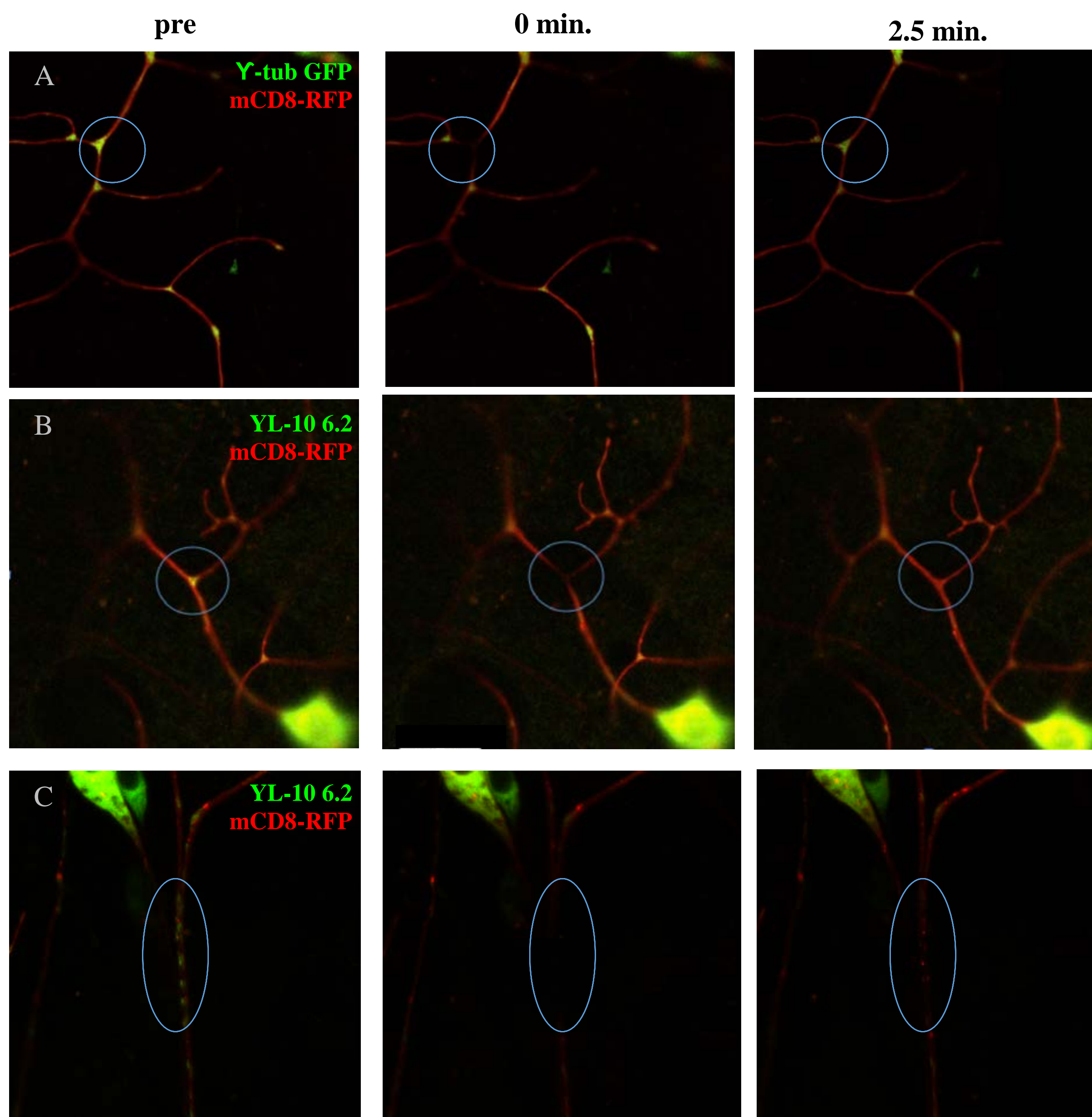


Figure 5. No recovery in ribosomal marker. FRAP was conducted on neuronal branch points and axonal segment. Images were taken before photobleaching, immediately after photobleaching, and 2.5 minutes post-bleach (A) ϒ-tubulin-GFP showed recovery of signal, but YL-10 ribosomal marker did not recover in dendritic branch point (B), nor in the proximal axon (C).

As seen, the ϒ-tubulin-GFP signal recovers (Figure 5A), but not theYL-10 signal (Figure 5B,C). Notice how even the RFP-tagged mCD8 recovers in this time. On the contrary, the YL-10 tag was not seen to recover at all. Thus, it was concluded that the marker was not tagging ϒ-tubulin and it was fully incorporated into the ribosome.

In addition, distal sections of separate axons were examined for ribosomal accumulations. This included the axonal boutons, which are the most distal parts of the axon (Figure 6):

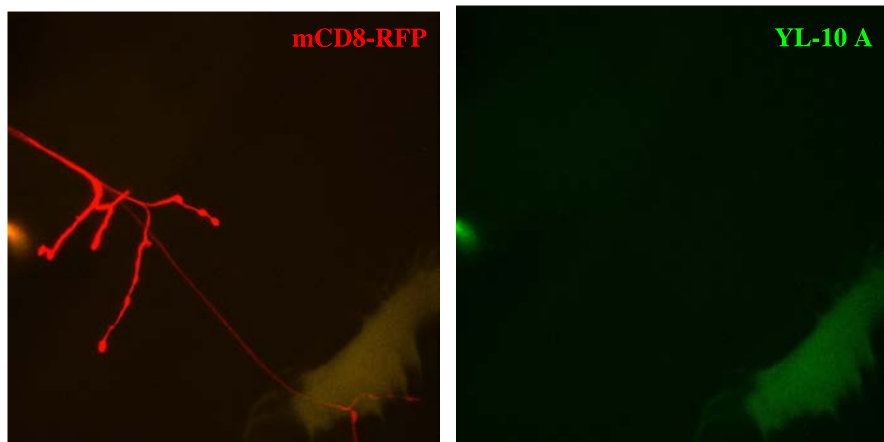


Figure 6. No ribosomal localization in distal axon. Separated channels of RFP and YFP show that there is no YL-10A in the distal boutons of the axon in motor neurons (Lauren Onweller, unpublished).

Though ribosomes are seen to accumulate in the proximal axon in *Drosophila* neurons, it was concluded that this was not the case in the distal axon of motor neurons. After confirming that this marker was tagging the ribosomes and that ribosomes did not accumulate in distal parts of the neuron, the YL-10 was validated and sufficient in examining ribosomal trafficking for following experiments.

Dynein knockdown shows decrease in dendritic ribosomes

I first aimed to find out if Dynein, the minus-end-out motor protein, played a part in ribosomal trafficking. This would ultimately suggest the importance of minus-end-out microtubules in this process. To uncover whether this motor protein was necessary for ribosomal trafficking, we utilized a Dynein RNAi. Using YL-10 to fluorescently tag ribosomes in class I *Drosophila* neurons, the localization of ribosomes in these knockdown neurons was examined and then compared to a control to assess localization differences when neurons lacked this motor protein. Since ribosomes accumulate in the branch points of dendrites, Dynein RNAi cells were expected to have significantly less ribosomes localizing at these branch points than a control. These direct results would help answer if minus-end-out motor proteins were responsible for the ribosomal trafficking throughout the neuron.

The cross utilized to study the effects of knocking down Dynein was as follows: **mCD8-RFP, Dicer2; 221:Gal4, UAS YL-10 A x Dynein RNAi**. This line contained YFP-tagged L-10 proteins for quantification of ribosomal accumulations, Dicer2 that allowed the RNAi silencing pathway[28], mCD8-RFP that allowed us to visualize the neuron, and the Dynein RNAi (Dhc64C) to knockdown the minus-end-out motor protein.

After this cross was set up, third instar larvae were mounted and an image of the individual class I neuron was taken. Control assays utilized reticulon (RTNL2) RNAi, a control genotype, in place of the Dynein RNAi. This allowed us to observe baseline cellular conditions when a random gene was knocked down; thus, any differences in ribosomal localization could be contributed to the knockdown of Dynein (Figure 7):

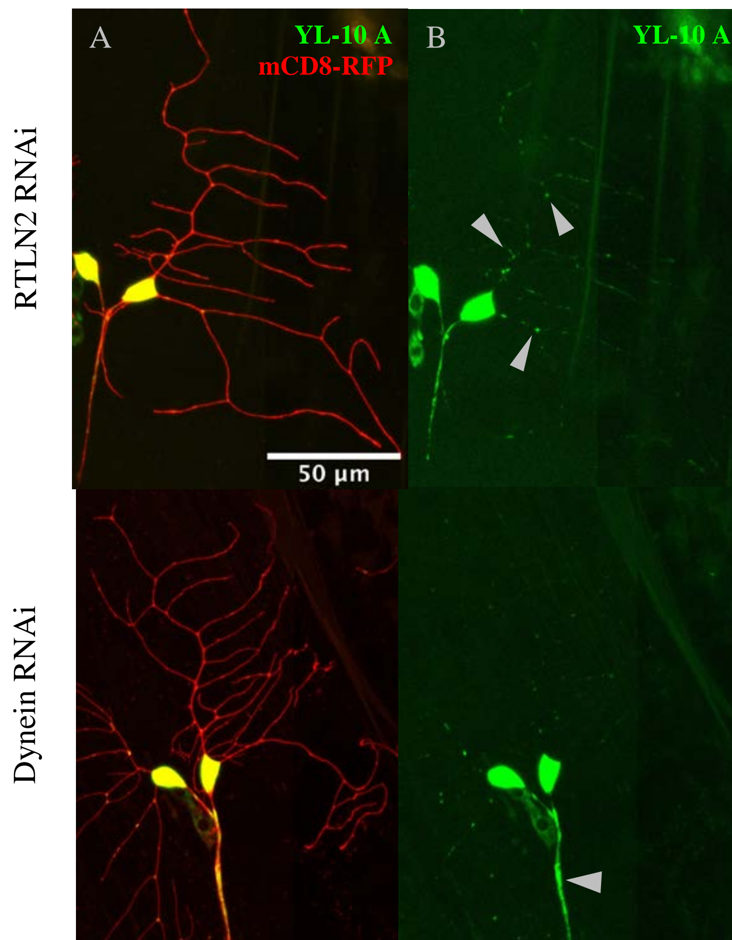


Figure 7. Effects of Dynein RNAi on ribosomal localization. (A) RTNL2 RNAi (control) still accumulates ribosomes in dendritic branch points. (B) Dynein RNAi has decreased ribosomal localization in the dendrites.

As seen in the images, there are visible accumulations of ribosomes in the dendritic branch points of the control neuron (Figure 7A). This seems to extremely decrease when Dynein is knocked down in the class I neurons (Figure 7B); there are barely traces of YL-10 signal in this scenario. This was further quantified to see if this was a significant decrease (Figure 8):

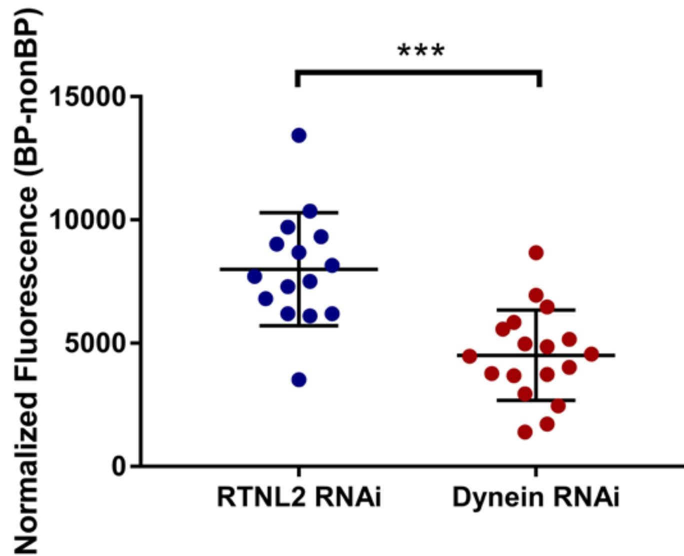


Figure 8. Dynein knockdown decreases dendritic ribosomal localization. The average branch point YL-10 A fluorescence was taken and normalized to non-branch point segments for each individual neuron. Dynein RNAi neurons (n=15) were calculated to have a significant decrease in this value compared to the RTNL2 RNAi control (n=18). ***P<0.0001 (unpaired t-test)

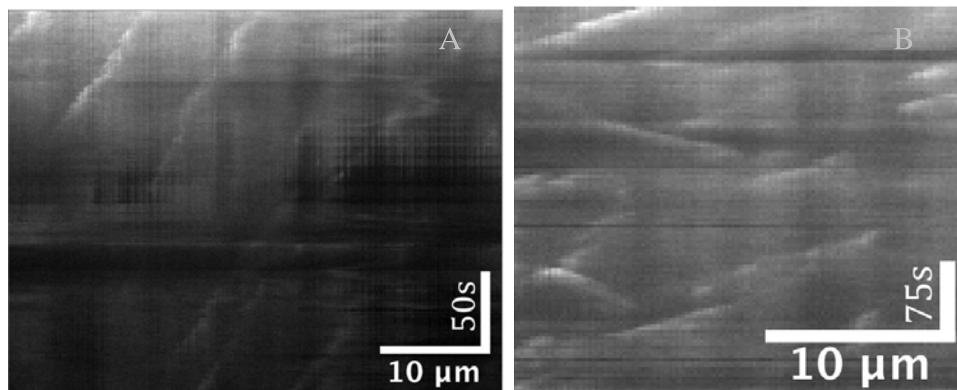
The results showed a significant decrease in ribosomes localizing to the dendrites when dynein was knocked down. This suggested that ribosomal localization is dependent on Dynein trafficking. It was also noticed that the YFP signal in the proximal axon seems to increase when dynein is knocked down (Figure 7).

Mixed polarity of the local axon results in increased ribosomal localization

Dynein facilitates the development of uniform plus-end-out polarity in the axon[29], and previous studies have observed that the knockdown of this motor protein leaves an accumulation of minus-end-out microtubules in the axon[30]. Hence, knocking down dynein yield mixed microtubule polarity in the axon, which left an environment for ribosomal to localize. Though

Dynein was previously suggested to be necessary for ribosomal trafficking, the proximal axon is potentially close enough to not need this motor protein for localization. I hypothesized that ribosomes would localize to the proximal axon under the Dynein knockdown due to the presence of minus-end-out microtubules.

Before examining localization in this scenario, microtubule polarity was monitored to support prior studies and confirm a non-uniform polarity in the axon. Recording the movement of EB1 tagged with GFP over time gives rise to several tiny "comets" that travel throughout the neurites. If the comets travel towards the cell, this indicates the presence of minus-end-out microtubules; if they travel away, this means the microtubules exhibit plus-end-out polarity. This was visualized by kymographs, which track the distance of each EB1-GFP comet from the cell body in respect to time, hence determining polarity in the studied neurite. Compared to the RTNL2 control, the Dynein knockdown neurons exhibited increased minus-end-out character in the axon. This was seen in both the kymograph of axon polarity and quantification across several different neurons (Figure 9):



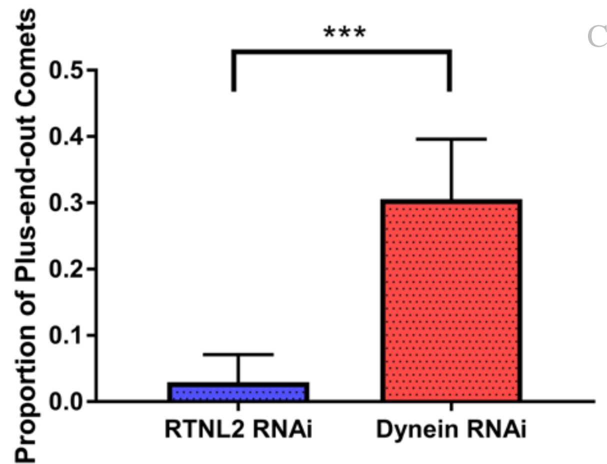


Figure 9. Dynein knockdown yields mixed polarity in axons. Kymographs tracking GFP-tagged EB1 were recorded measuring time vs. distance from the soma. Compared to uniform polarity in the axon in RTNL2 RNAi control neurons (A), Dynein knockdown axons exhibit mixed polarity (B). This was further quantified (C), where the proportion of minus-end-out character for each axon was visually determined via EB1 comet assays. ***P<0.0001 (unpaired t-test)

As seen in both the kymographs and visual comet counts, Dynein RNAi neurons exhibit mixed polarity in the axon. This differs from the established uniform plus-end-out polarity in the control RTNL2 neurons. Once the non-uniform axonal polarity was confirmed in the Dynein knockdown, we then examined ribosomal localization in the proximal axon. YL-10 signal was quantified in the first 75 microns (µm) of the axon to see if the polarity had an effect on this localization (Figure 10):

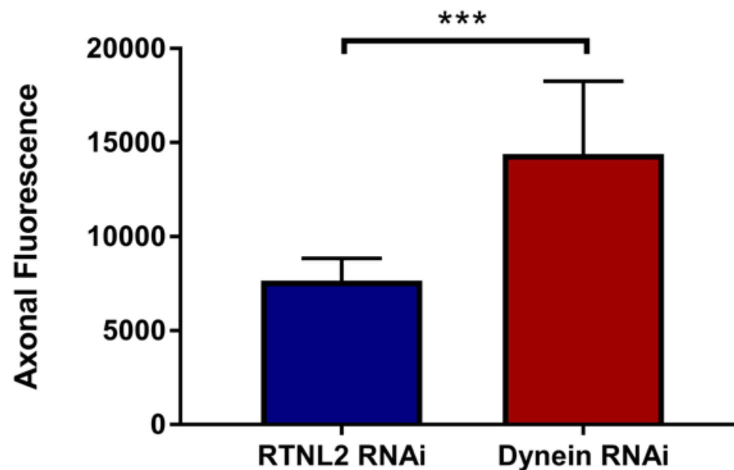


Figure 10. Knockdown of Dynein yields increased ribosomal localization in proximal axon . Under the knockdown of Dynein (n=11), in which minus-end-out microtubules were present in the axon, there was a significant increase of ribosomes compared to the RTNL2 control (n=11). ***P<0.0001 (unpaired t-test)

Here, we determined that there was a distinct increase of YL-10 signal in the Dynein RNAi compared to the control. This increase of ribosomal localization in the axon occurs when there is mixed polarity in this part of the neuron. While this project has primarily examined the dendritic branch points to understand ribosomal localization, this was yet another way to suggest the hypothesis that ribosomes localize to minus-end-out microtubules.

Plus-end-out microtubule polarity yields decreased ribosomal localization in Patronin RNAi

At the forefront of minus-end-out microtubule stability lie specific proteins that bind the long microtubule polymers. Calmodulin-regulated spectrin-associated proteins (CAMSAPs) are responsible for the specific minus-end stabilization in vertebrates[31]. These act to bind the ends of microtubules and prevent these structures from dissociating or breaking down. In addition,

Patronin is responsible for binding minus-end-out microtubules in *Drosophila* to stabilize them and facilitate growth. Previous studies show that Patronin binds selectively to the minus-ends of microtubules and protects them from Kinesin-13-induced depolymerization [32]. An interesting phenotype has been uncovered when this crucial protein is depleted in neurons. Our lab has shown that the knockdown of this protein causes a combination of minus-end-out and plus-end-out microtubule polarity in class IV *Drosophila* dendrites (Pankajam Thyagarajan & Chenye Feng, unpublished). This leaves a neuronal model in which the presence of plus-end-out microtubules in the dendrites can be tested. Here, differences in ribosomal localization were examined between branches with different microtubule polarities.

I hypothesized that dendritic branches maintaining original microtubule polarity in the Patronin RNAi model would accumulate ribosomes, but not branch points exhibiting plus-end-out polarity. The affect of Patronin RNAi on microtubule polarity (Figure 11) allowed us to take examine the effect of different branch point polarities on ribosomal localization:

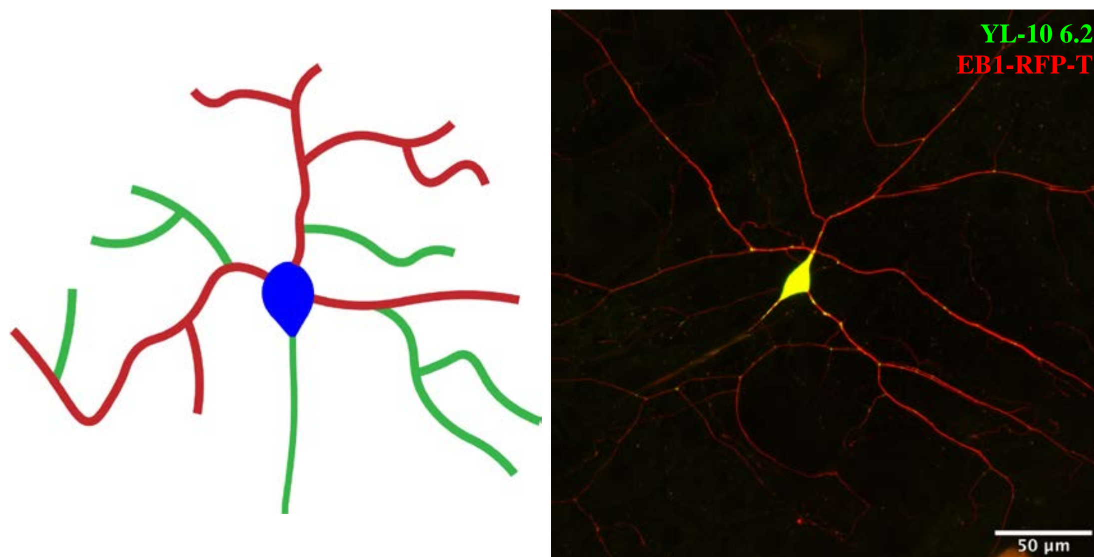


Figure 11. Patronin RNAi polarity in class IV neurons. Class IV neurons exhibit mixed microtubule polarity when Patronin is knocked down. This allows the comparison of ribosomal localization in branch points that are characterized as minus-end-out (red) and plus-end-out (green).

We utilized a fly line that included a pickpocket (ppk) driver for expression in class IV neurons, EB1-RFP-T tag to visualize microtubule polarity, and Patronin RNAi (ssp4, BL36659) for disrupting dendritic polarity. This line was crossed to the YL-10 6.2 line to visualize ribosomal localization in class IV *Drosophila* neurons. After the final cross was set up, third instar larvae were imaged. From these analyses, association between minus-end-out polarity and ribosomal localization could either be supported or refuted.

Localization of ribosomes and microtubule polarity were examined simultaneously. First, a Z-stack image was taken to view ribosomal localization, which was measured for YL-10 intensity at the branch points. Then, a time-lapse video of the cell was taken to examine the movement of the tagged EB1 proteins. If ribosomes localized to dendritic branch points of minus-end-out polarity and not plus-end-out polarity, this would suggest that the localization is based on minus-end-out microtubules.

Branch points were categorized as “minus-end-out” or “plus-end-out” based on the comet assays of each dendrite. To determine whether a branch point was considered minus-end-out or plus-end-out, the immediately proximal dendrite branch and branch point was checked for polarity. Dendrites exhibiting greater than 60% minus or plus-end-out polarity were utilized in this study. This cutoff was optimal for the sample size of dendrites studied and allowed for branch points to have an established polarity. The ribosomal localization of such branch points were compared to yield interesting results (Figure 12):

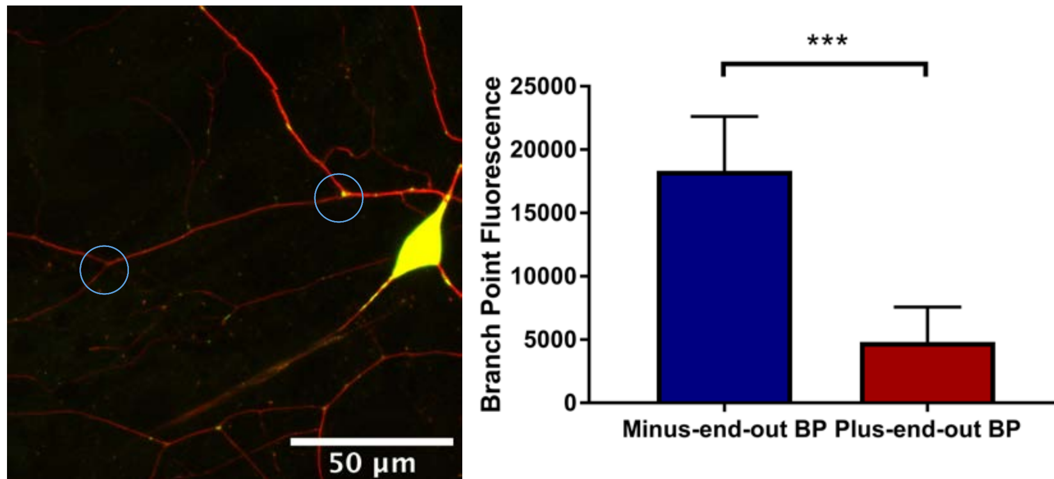


Figure 12. Effects of Patronin RNAi on ribosomal localization. Dendritic branch points were defined as either “minus-end-out “(>60% minus-end-out character, n=6) or “plus-end-out” (>60% plus-end-out character, n=10). These were examined for then quantified for ribosomal intensity. ***P<0.0001 (unpaired t-test)

A significantly lower level of ribosomes was seen to localize to branch points that were categorized as plus-end-out. These results further supported the hypothesis that ribosomes are transported along minus-end-out microtubules.

Axon injury confirms decrease in ribosomal localization in branch points with opposite polarity

Finally, axon regeneration was utilized to study ribosomal localization in class I *Drosophila* neurons. Studies show that after completely severing the axon of a neuron, one of the cell’s dendrites completely reverses its polarity, becoming the new plus-end-out axon[33]. This holds true in axons cut within 35 µm of the soma, compared to axon regrowth when the cut is induced outside this parameter[34]. I hypothesized that ribosomal localization would decrease in dendritic branch points that flipped polarity to become the new axon post-injury.

First, axotomy was performed by a UV pulse laser and the axon was cut off; then, the regeneration process began. In class I ddaE neurons, regeneration is usually selected to occur in the non-comb dendrite[33]. So, samples in which the horizontally extending non-comb dendrite converted to the new axon were analyzed to yield consistent results. Within 24 hours, microtubule polarity in the converting axon is mixed and on its way to purely plus-end-out[33]. This is accompanied by the growth of this neurite into the long structure of the axon. Finally, an established plus-end-out microtubule polarity is seen 72 hours after cut. The conversion of the new axon from the non-comb dendrite is complete (Figure 13):

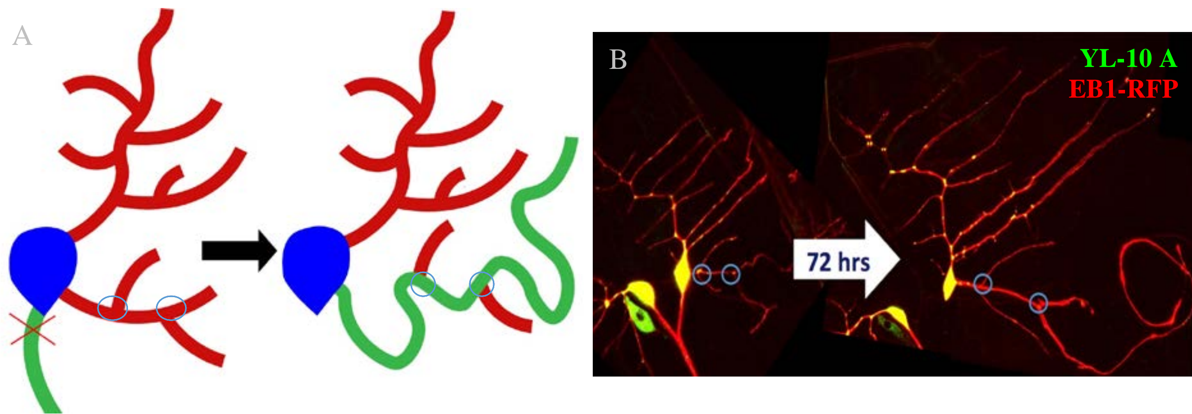


Figure 13. Axon regeneration after induced injury. (A) The axon of a class I *Drosophila* neuron is cut in the proximal axon. This is followed by the conversion of the non-comb dendrite flipping its polarity from minus-end-out (red) to plus-end-out (green) and the outgrowth of this new axon. (B) *in vivo* axon regeneration showing class I neuron precut and 72 hour post-cut.

The non-comb dendrite that converted into the new axon exhibited plus-end-out microtubule polarity post-axotomy. Here, we could see if this conversion yielded changes in ribosomal localization in these branch points (circled, Figure 13B). First, microtubule polarity in both pre-cut and post-cut neurons was examined to confirm that the axotomy caused flip in polarity via EB1-GFP comet assays. Here, the three distinct sections of the non-comb dendrite were studied: the primary non-comb dendrite, the secondary branches extending from this

primary dendrite, and the distal tip of the dendrite that grew out when converted to the axon. This was then compared with the ribosomal localization in these branch points to see if ribosomal trafficking was dependent on the original minus-end-out polarity (Figure 14):

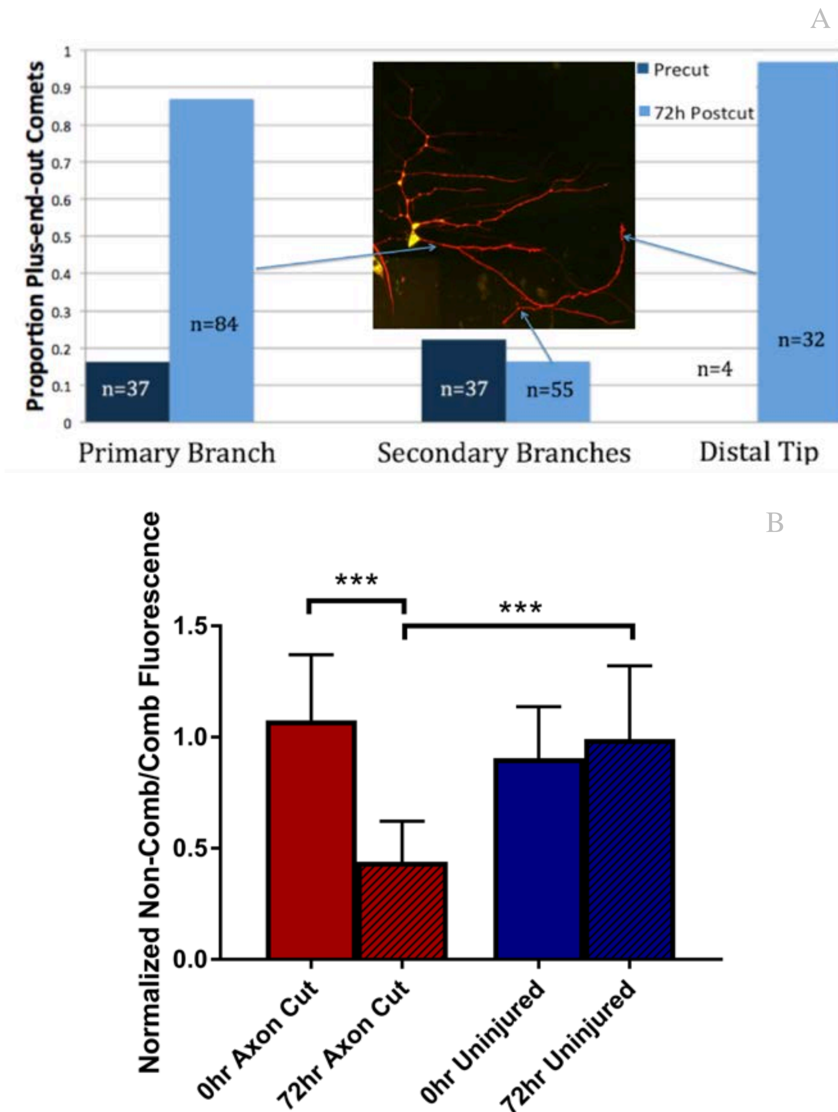


Figure 14. Polarity flip in new axon yields decrease in ribosomal localization. (A) Comet assays were conducted on dendrites of pre-cut and post-cut neurons. All parts of pre-cut dendrites were seen to have a mostly minus-end-out polarity, as expected. This was almost completely flipped to plus-end-out after the conversion to new axon in both the primary branch and growing distal tip. Sample size (n) represents the number of EB1-GFP comets measured. (B) Dendrites that converted to the new axon have significantly decreased ribosomal localization post-cut (n=10). Uninjured neurons (n=11) maintained a steady level of ribosomal localization over 72 hours. ***P<0.0001 (unpaired t-test)

As expected, the dendrites exhibited minus-end-out microtubule polarity before axotomy, which validated past findings (Figure 14A). When the non-comb dendrite was converted to the new axon, this microtubule polarity was completely flipped to plus-end-out, which was characteristic for an axon. Each branch point in which the primary non-comb dendrite extended a secondary branch was measured for ribosomal localization. To keep results consistent across samples, this was internally normalized to comb branch points that did not convert microtubule polarity. Interestingly, a significant decrease in ribosomal localization occurred in non-comb branch points after they flipped polarity (Figure 14B). These findings support the hypothesis that ribosomal localization is dependent on minus-end-out polarity.

Chapter 3

Discussion

This work suggests that ribosomal localization is directly correlated with minus-end-out microtubule polarity. As shown in localization assays and previously established in the literature[17], ribosomes were present in neurites containing minus-end-out microtubules. This was not only true for the established dendrites, but also the proximal axon in when Dynein was knocked down and mixed polarity was observed. Other experiments were utilized to compare ribosomal localization in dendrites containing natural minus-end-out polarity with these same branch points when plus-end-out polarity was induced. Our studies have not only examined how ribosomal trafficking is affected when a potential transporting motor protein is knocked down in neurons, but they have also inspected how altered microtubule dynamics impacts the trafficking of ribosomes.

The impact of these conclusions on the field of biology is widespread. This dataset can help draw conclusions on the ongoing question whether or not the minus-end-out axon accumulates ribosomes or is home to protein synthesis. In addition, experimentation can be applied across other model organisms for confirmation that ribosomal trafficking is conserved. This information and experimentation yields insight on protein specification and translation in certain compartments of neurons, as well as suggestions on how other cellular components localize specifically to dendrites. It has been established that knocking down Dynein significantly decreases ribosomal localization in the dendrites. This suggests that the motor protein is necessary for ribosomal trafficking to the dendrites (Figure 16):

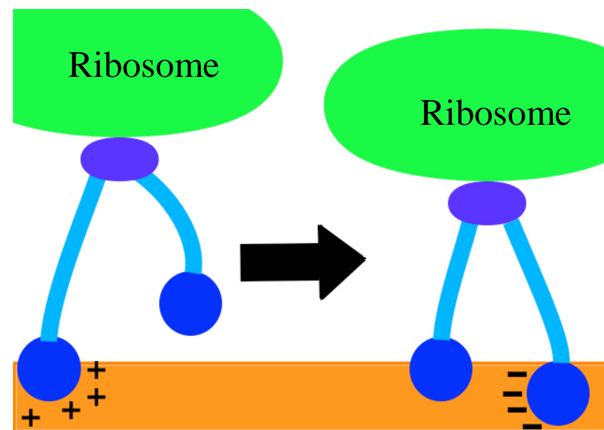


Figure 15. Suggested model of ribosomal trafficking via Dynein. Dynein motor protein travels along microtubules in the minus-end-out direction. Studies suggest that Dynein is necessary for ribosomal localization to dendritic branch points, thus are trafficked as cargo along minus-end-out microtubules.

These results can lead to a plethora of follow-up studies, in which both cellular trafficking and protein synthesis can be examined. Foremost, the procedures utilized to test ribosomal trafficking can be equally as useful in determining the trafficking of other important neuronal components. Like ribosomes, the rough ER and Golgi complex have been seen to accumulate in the soma and dendritic branch points of *Drosophila* neurons[23]. Experiments in which uniform minus-end-polarity is altered and Dynein is knocked down can potentially suggest that these organelles are trafficked in the same nature as ribosomes. Fly lines have been established, like UAS-ManII-eGFP[35] and BiP–sfGFP–HDEL[36], to examine the localization of Golgi and rough ER, respectively. I would hypothesize that similarly to ribosomes, the trafficking of these organelles would be dependent on minus-end-out microtubules.

Localized dendritic translation can also be studied with the newfound understanding of ribosomal trafficking, too. This can be especially useful in understanding dendrite degeneration and regeneration. While dendrite regeneration has been studied, is not completely understood[37].

Inducing dendrite injury under a knockdown of Dynein can suggest whether local translation is necessary for dendrite regeneration. I hypothesize that local translation of protein is needed for dendrite regeneration, and the lack of trafficking to these neurites would inhibit this regeneration. Furthermore, the role of local translation in dendrite degeneration can be studied using these findings. Fidgetin, a microtubule severing protein, promotes dendrite degeneration following dendrite injury. It has been shown that this degeneration was delayed under conditions where fidgetin was depleted via deficiency mutations or RNAi[38]. Whether or not dendrites properly degenerate under the Dynein RNAi can suggest if this important severing protein is locally translated. Since fidgetin specifically functions in dendrite degeneration, I hypothesize that this is locally translated in these branches and degeneration would be delayed when Dynein is knocked down.

When it comes to life quality in all humans, the maintenance of neurons is of utmost importance. The nervous system is central to virtually every function in the body, holding true in specimens from fruit flies to complex vertebrates. Understanding trafficking means of cellular components can lead to a better grasp of neuronal function, helping to understand the body and lead to therapeutically combat neurodegeneration in the long run. Specifically understanding why dendrites, but not the axon, house ribosomes can help explain the differential response to trauma in these neurites. Protein synthesis in neuronal dendrites has been widely studied and established as an important aspect in establishing the local neuronal proteome[39]. Thus, trafficking of the ribosome to specific regions of the dendrite remains an important aspect in healthy organisms.

Chapter 4

Methodology

Fly stocks, crosses, and housekeeping

Each fly line was individual stored in a plastic bottle, containing a substantial amount of fly food and capped with a cotton plug to allow the escape of air. The food was made of a mixture of water, agar, yeast, soy flour, yellow cornmeal, light corn syrup, and propionic acid. These fly bottles were stored at a constant temperature of 25°C and flipped roughly every 3 weeks to avoid contamination and mite accumulation.

To obtain our desired cross, desired genotypes were then stored at 18°C and monitored for virgin female flies to hatch. These were collected and crossed to the adult male of another line. Crossed flies were kept in an isolated bottle at 25°C, and the food caps were changed every day. The third instar larvae were able to be isolated from each cap for microscopy experiments.

Fluorescent protein expression in class I and IV neurons

The Gal4/UAS system was utilized to label proteins of interest in specific *Drosophila* neurons. For decades, this binary expression tool has made *Drosophila* an optimal model organism for microscopy. The yeast transcription factor Gal4 is produced, which then binds to the upstream activation sequence (UAS) and allows the direct expression of the desired upstream fluorescent protein[40]. Different *Drosophila* da neurons were examined in different experiments, which meant different drivers were required. Gal4 expression was driven by 221 in Class I ddaE

neurons, while pickpocket was the Gal4 driver in Class IV ddaC neurons. Both drivers have been previously established to have strong expression in each specific neuron subclass[41].

RNAi in assays

The method of knocking down genes in *Drosophila* has been extensively studied, and results demonstrate that transgenic RNA interference (RNAi) is potent in neurons, with significantly low false-negative rates[42]. This knockdown of gene expression occurs at the post-transcriptional level. Small interfering RNA (siRNA) for the protein being targeted is produced and forbids the translation of the specific mRNA. The protein Dicer was incorporated in these fly crosses, as well as the RNAi sequence of interest. Dicer functions to cut the siRNA, which is then incorperated to the RISC complex. Here, the mRNA of the knockdown is targeted and not able to be translated[43]. In addition to each desired knockdown, an additional control RNAi was incorporated. This was a reticulon 2 (RTNL2) RNAi, which does not have any noticeable effect on the *Drosophila* neurons.

Live imaging of ribosomal localization and microtubule dynamics

Imaging of each neuron was conducted on a Zeiss LSM confocal or Zeiss Widefield microscope. An individual fly larva was washed in water, and then placed on a microscope slide spotted with dried agar. The larva was then clamped with a cover slip, and oil was applied to allow 63X oil lens magnification.

To determine ribosomal localization, settings were optimized to allow detection of both YFP and RFP. The L-10 protein of ribosomes were tagged with yellow fluorescent protein (YFP), which closely resembles green fluorescent protein (GFP). The YL-10 was excited at 488 nm and emitted signal at 510 nm. To examine the structure of each neuron, and even morphological changes post-axotomy in axon cut assays, proteins tagged with red fluorescent protein (RFP) were utilized. Cellular markers, including mCD8 and EB1, allowed the visualization of the desired neuron; the RFP was excited at 546 nm and emitted at 584 nm.

To determine microtubule dynamics in the axons and dendrites of specific cells, EB1 assays were performed. This utilized the established procedure of fluorescently tagging end binding protein 1 (EB1) to monitor the direction microtubules grow in specific parts of the neuron[44]. Over a five minute screening, images were taken every second to examine movement of the EB1 in a time-lapse video. When the tagged EB1 spots, or "comets", travelled away from the cell body, this was indicative of plus-end-out microtubules. Plus-end-in, or better known as minus-end-out, microtubules are identified as the EB1 comets travelling toward the neuronal cell body.

Axon injury

Cutting the proximal axon of neurons is a powerful assay and has been extensively studied to better understand this unique cell type[33]. A pulsed UV laser attachment was utilized on the Zeiss LSM confocal. By pressing a foot pedal, the system was triggered to fire a laser at the scoped out portion of a neuron, in this case the proximal axon. These were clean cuts, and

explosion cutting was avoided. It was made sure that the axon was completely severed from the soma, and then the larva was isolated for future examination.

Data collection and quantification

Each localization image was taken as a Z-stack, to ensure ribosomes were accounted for in each horizontal plane of the neuron. After collecting the image, it was imported to the ImageJ analysis software. From here the Z-Stack was collapsed to a single, quantifiable image. Each analyzed dendritic branch point was traced, then YFP signal intensity was quantified and recorded. The microscope's laser intensity was adjusted to make sure there was no saturation in the YFP signal.

In EB1 comet assays, the 300-frame time-lapse video was sped up to roughly 20 frames per second for analysis. The direction of each visual EB1 dot was visually determined and compared with others in the specific neurite, then the microtubule polarity of each section was determined. In addition, kymographs were constructed to confirm findings. These visual figures mapped out EB1 distance travelled versus time.

Each captured picture of the class I and IV neuron was analyzed on ImageJ software utilizing the intensity tool. Here, ribosomal intensities were measured in both specific dendritic branch points and proximal axons, and the dynein knockdown data was compared to the control.

Statistical analysis

A standardized t-test was utilized to compare data points from separate groups. P values were calculated and were represented by the following denotation: *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$. T-tests were run and p values calculated on GraphPad Prism 9 software.

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University of Pittsburgh Summer Undergraduate Research Program Summer 2018
Dr. Patrick Pagano- Vascular Medicine Institute at the University of Pittsburgh
- Utilized cell culture and molecular pharmacology to examine a specific NADPH Oxidase 5 (Nox5) peptide inhibitor in attempt to combat vascular disease
- Awarded 3M Fellowship to continue research for two additional weeks

UNC Summer of Learning and Research (SOLAR) Program Summer 2017
Dr. Lisa Tarantino- University of North Carolina at Chapel Hill
- Examined the genetics underlying cocaine addiction using a mouse model
- Actively participated in professional development workshops, GRE test preparation, and STEM classes, all while conducting full-time research

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Dr. Melissa Rolls- The Pennsylvania State University
- Awarded fellowship to continue research full-time throughout the summer
- Worked on my thesis and gained research experience

Experience:

Start-up for STEM education Fall 2018
Happy Valley LaunchBox

- Selected to participate in rigorous program with hopes to build a business
- Learning how to go about entrepreneurship and catering to potential customers

Schreyer Ambassador Team (SAT) Fall 2017- Present
Penn State Schreyer Honors College
- Serving as a student ambassador and representative for the Schreyer Honors College
- Working with a team of other like-minded students to plan events, give tours, raise money, and connect with donors and alumni

Cohort Council Representative Fall 2018- Present
Millennium Scholars Program
- Chosen by peers to represent the 3rd Cohort of the Millennium Scholars Program
- Meet with administration and work to excel the program

THON Fall 2016- Present
Apollo (Special Interest Organization)
- Supporting Penn State's dance marathon that benefits The Four Diamond Fund for pediatric cancer
- Serving as trip leader for fundraising, supporting our THON families, and raising awareness for pediatric cancer

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Penn State Undergraduate Research Symposium	Spring 2016, 2017, & 2019
Eberly College of Science Benefactor Recognition Dinner	Fall 2017
University of North Carolina Research Symposium	Summer 2017

Extracurricular Activities:

American Society for Pharmacology and Experimental Therapeutics	2018-Present
National Society of Leadership and Success	Spring 2016- Present
Millennium Society	Fall 2015- Present
Penn State Blue & White Society	Fall 2015- Present
Independent tutoring of Calculus I	Summer 2018
Schreyer Honors College Student Council	Fall 2015- 2017
Undergraduate Research Society	Fall 2015- 2016

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Hyman and Jennie Burstein Memorial scholarship
Academic Excellence scholarship
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