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Understanding how Cyclin-Dependent Kinase 5 Regulates Microtubule Polarity in Class I Dendritic Arborization Neurons of *Drosophila*

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ABSTRACT

Proteins constituting the Wnt signaling pathway—γ-tubulin, Axin, Dsh, and Fz—regulate microtubule polarity in the dendrites of class I dendritic arborization (DA) neurons in *Drosophila.* Due to the distinct polarity of microtubules, microtubule motor proteins—dynein (minus end directed) and kinesin (plus end directed)—can ensure correct transport of cellular cargo within the cell. Within the dendrites, microtubule are oriented plus ends towards the cell body and minus ends directed away from the cell body. Previous studies indicate the Wnt proteins organize microtubule organization centers (MTOCs) to dendritic branch points in class I DA *Drosophila* neurons—this results in nucleation and maintenance of microtubules in the cell. Additionally, Cyclin Dependent Kinase 5 (Cdk5) exhibits important regulatory roles in neuronal cells. Given this, we aimed to study the role Cdk5 plays in regulating microtubule polarity in class I DA *Drosophila* neurons. We developed two alternate hypotheses to address our overarching question: H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 4). Our experimental evidence showed depletion of Cdk5 in the cell resulted in decreased localization of γ-tubulin to dendritic branch points, and mixed microtubule polarity in the cell. Exploration of the localization patterns of various proteins involved in localizing γ-tubulin to dendritic branch points in class I DA *Drosophila* neurons—Axin, Dsh, APC2, and Fz—following knockdown of Cdk5 in the cells supports hypothesis 1: Cdk5 appears to act on proteins upstream of Axin within the Wnt signaling pathway. Furthermore, fluorescence microscopy experiments revealed Cdk5 localizing to the cell body of class I DA *Drosophila* neurons, which indicates Cdk5 could have other cellular functions, in addition to regulating microtubule dynamics.

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Chapter 1

Introduction

Sub-Chapter 1: Cytoskeletal Proteins in Healthy Neurons

Neuron and Cytoskeletal Protein Overview

Neurons are postmitotic cells required for cell communication within the nervous system. Most neurons contain a basic structure; the cell body, the dendrites, axons, and axon terminals (Figure 1). Cytoskeletal proteins regulate healthy neuronal function. Some notable cytoskeletal proteins are actin (protein subunit for microfilaments) and tubulin (protein subunit for microtubules). The cytoskeleton is essential for maintaining cell shape, compartmentalizing the cell, and maintaining the compartmentalization of biochemical processes, which can have a critical downstream effect such as controlling gene expression, cell differentiation, proliferation, and survival (Verin, A.D, Bogatcheva, N.V., 2006). In addition to these functions, the cytoskeleton is capable of undergoing rearrangement allowing for cell motility, contraction, and morphogenesis (Verin, A.D, Bogatcheva, N.V., 2006). Understanding the importance of cytoskeletal proteins in maintaining healthy neuronal functions can drive us to study proteins responsible for regulating these proteins and develop our understanding of healthy and disease-state neurons.

Cytoskeletal Protein: Microtubules

Microtubules are a critical cytoskeletal protein necessary for maintaining proper neuronal function throughout an organism's life. Microtubules are hollow and dynamic cytoskeletal proteins important for transferring cargo throughout the cell composed of alternating α-tubulin and β-tubulin heterodimer subunits (Cooper GM., 2000). Regarding microtubules, distinct polarity shows plus-ends associated with the polymerizing end, the location of the β-tubulin. In contrast, the minus-end associates with the depolymerizing end, the site of α-tubulin (Cooper GM., 2000). Microtubules are "train tracks" that allow cargo to be moved throughout the cell with cues from specific motor proteins that recognize distinct polarity in these cells. Microtubule polarity cytoskeletal proteins can be important for distinguishing the axons from the dendrites. The microtubules' polarity is essential to ensure that proteins can determine various cell parts to transport cargo to correct cell locations. Particularly, dynein motor proteins navigate intracellular transport towards minus-ends of microtubules (Paschal, B.M. & Vallee, R. B. 1987) (Schroer, T. A., et al. 1989). Also, dynein localizes near the nucleus in the microtubule organizing centers (in post mitotic cells) (Roberts, A. et al. 2013). On the other hand, kinesins serve vital roles for cellular transportation along microtubules. Kinesins are responsible for plus-end directed transport and other anterograde transportation—transporting secretory vesicles to the plasma membrane and radial movements of the endoplasmic reticulum membranes (Lodish, H., et al. 2000). In neurons, microtubules are dynamic and have an inherent polarity. More specifically, in developing *Drosophila* dendrites, there is evidence of mixed microtubule polarity (Hill SE. et al., 2012).; however, microtubules in the dendrites of mature dendritic arborization *Drosophila* neurons have predominately minus end-directed away from the cell body (Rolls M. M., Satoh D., et al., 2007) (Figure 1). There is evidence of similar minus-end out organization in other model

systems such as *C. elegans* (Goodwin PR, Sasaki JM., et al., 2012). In contrast, the axons of *Drosophila* DA neurons exhibit plus-end out (away from the cell body) microtubule polarity (Rolls, M. M. et al. 2007) (Stone, M. C., et al. 2008). Given the minus-end out polarity pattern in mature *Drosophila* dendrites, there is evidence that the minus-end directed motor protein, dynein, plays an essential role in the transport of cellular components from the cell body out to the mature dendrites (Satoh D. et al., 2008), (Zheng Y. et al., 2008). In contrast, given the mixed-microtubule polarity present in developing *Drosophila* dendrites, plus end-directed kinesins could play a role in cargo transport into both axons and dendrites (Namba, T. et al., 2011) (Kapitein LC, Hoogenraad CC, 2011).

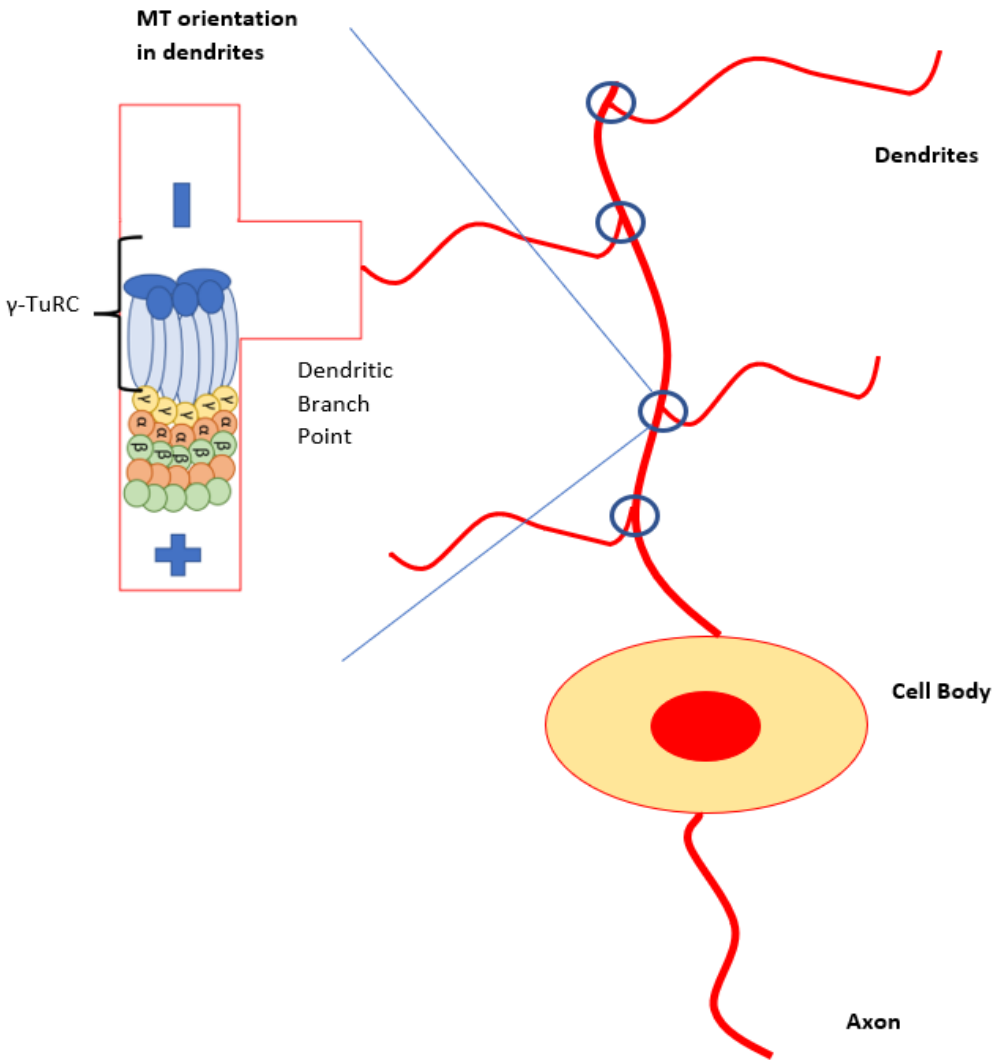

Figure 1. General overview of a neuron; Axon, Cell body, and Dendrites. Blue circles indicate that the γ-tubulin ring complex, responsible for nucleation and regulation of microtubule dynamics, localizes to the dendritic branch points. This figure represents a simple model of a class I *Drosophila* neuron. The dendrites, cell body, and axon are labeled next to their representative structure. The blue circles indicate the location of microtubule organization centers (MTOCs) and show the polarity of microtubules found within the dendritic branch points (plus sign oriented towards the cell body and minus end oriented away from the cell body).

Regulators of Microtubule Organization Centers (MTOCs)

A key regulator of microtubule dynamics is the microtubule organization center (MTOC), which regulate the nucleation, assembly, and polarity of microtubules. γ-Tubulin is a third type

of tubulin subunit and is an essential protein component of the γ-tubulin ring complexes (γTURCs) responsible for regulating the dynamics of microtubules in cells (Zheng et al., 1995). γ-tubulin, a protein of the γTURCs, is known to concentrate at dendritic branch points in *Drosophila* (Nguyen MM. et al., 2014) (Figure 2). Proteins constituting the Wnt Signaling pathway are responsible for localizing γ-tubulin to dendritic branch points to regulate microtubule polarity and nucleation of microtubules (Figure 2). More specifically, the seven-transmembrane protein Wnt receptor, frizzled (Fz), a Wnt coreceptor, arrow (arr), heterotrimeric G-proteins, disheveled (Dsh), casein kinase I $(CK1)_{\gamma}$, and Axin seem to be sufficient in localizing γ-Tub to dendritic branch points (Weiner A.T. et al., 2020). Studying how y-tubulin and other proteins that constitute the Wnt signaling pathway (Axin, Dsh, and Fz) localize to dendritic branch points can lead to a better understanding of neuronal function in healthy and diseased neurons.

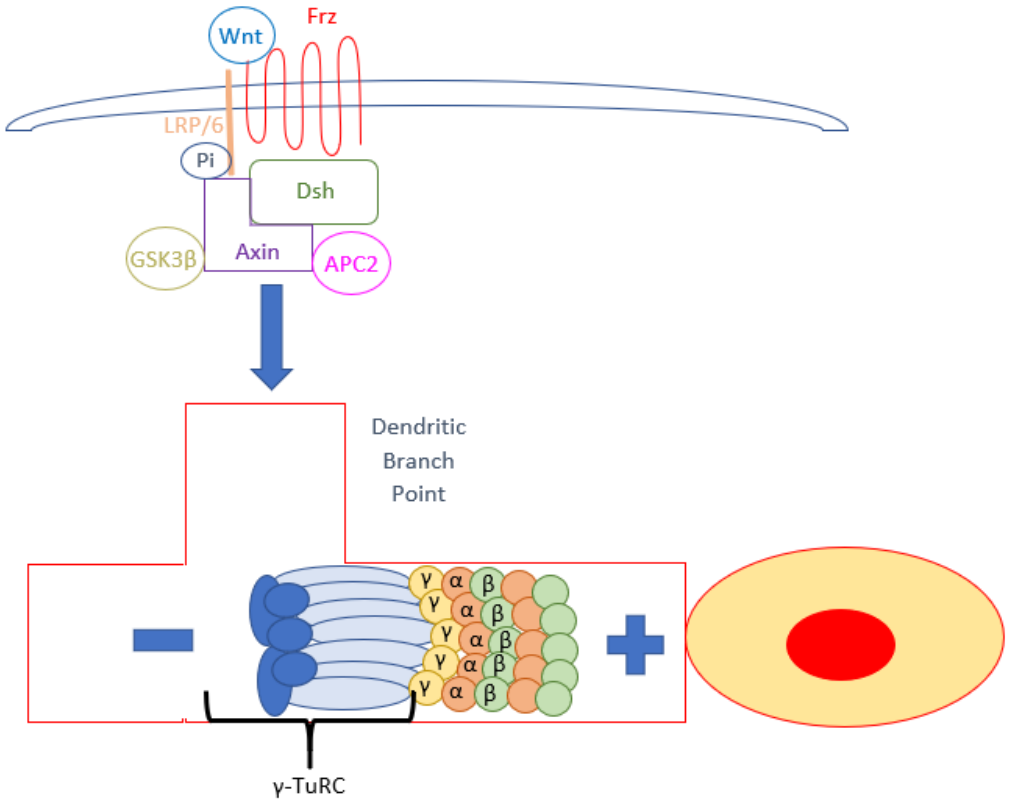

Figure 2. The Wnt signaling proteins, which constitute the Wnt Signaling pathway, are responsible for localizing γ-tubulin to dendritic branch points. This image displays the Wnt signaling proteins in the presence of the Wnt protein. The blue arrow represents the Wnt proteins ultimately responsible for microtubule nucleation and regulation within dendritic branches. The plus and minus sings represent the microtubule polarity in the dendrites (plus end oriented towards the cell body and minus end oriented away from the cell body).

Sub-Chapter 2: Cyclin Dependent Kinase 5 (Cdk5) Regulation of Neurons

Unique Characteristics of Cdk5

Cyclin-Dependent Kinase 5 (Cdk5) is a unique, postmitotic, serine/threonine kinase, initially classified with other CDK family members based on sequence homology (Zelda H. Cheung and Nancy Y. Ip, 2004). Cdk5 is distinct from other cyclin-dependent kinases in that it is activated in neuronal cells while other cyclin-dependent kinases are inactive (Shah K and Lahiri D. K, 2016). The latter, family of kinases, have important roles in cell cycle progression by mediating mitosis and cell movement along microtubule tracts (Zelda H. Cheung and Nancy Y. Ip, 2004). Cdk5 does not appear to play a role in cell cycle progression but seems to play a role in maintaining neuronal survival and neuronal death. The function of Cdk5 changes throughout the life of the cell. From playing a role in brain development in infancy to other functions later in cellular life such as regulating neuronal survival, enhancing synaptic plasticity, facilitating learning and memory formation, regulating pain signaling, contributing to drug addiction, and influencing long term behavioral changes (Grother MJ et al. 2018) (Figure 3). When active, Cdk5 phosphorylates various cytoskeletal components and signaling molecules (Nikolic et al., 1998) (Keshvara et al., 2002). The phosphorylation of these target proteins allows for proper neuronal migration, neurite outgrowth, cerebellar formation, and survival (Nikolic et al., 1996) (Ohshima et al. 1996) (Oshima et al., 1999) (Tanaka et al., 2001).

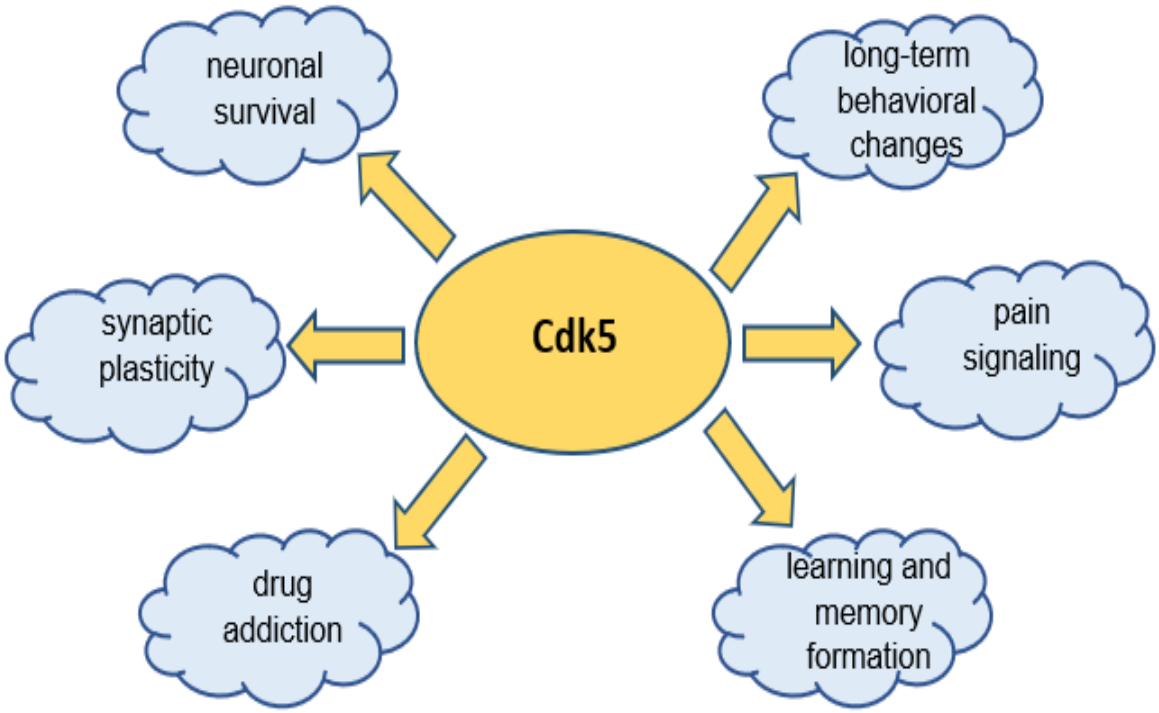

Figure 3. Cdk5 regulates functions in the cell that control our learning and memory formation, pain signaling, drug addiction, synaptic plasticity, long-term behavioral changes, and overall neuronal survival. This figure displays key functions Cdk5 maintains in neuronal cells.

Cdk5 Role in Neuronal Survival and Neuronal Death

Given the understanding of the roles Cdk5 plays in the brain, it is not surprising to find the significant roles it has in maintaining healthy neurons by promoting neuronal survival and promoting neuronal cell death. In Cdk5 knockout mice, there were lesions present in the central nervous system and accumulation of neurofilament immunoreactivity, which indicates Cdk5's role in neuronal differentiation and neuronal cytoskeletal structure organization (Ohshima T. et al. 1996). Reintroduction of Cdk5 into the Cdk5 null mice reversed the observed adverse phenotypes, which further demonstrated the role of Cdk5 in healthy development and survival of neurons (Tanaka T. et al. 2001). Furthermore, knockout of Cdk5 in mice also indicates the role of Cdk5 in the regulation of neuronal cell death pathways by inhibiting c-Jun N-terminal kinase

3 (JNK3) activation (Li B.S. et al. 2002). An additional study has shown the involvement of Cdk5 in maintaining survival signals for neurons through the PI3K/Akt pathway (Li B.S., et al. 2003). Additionally, a study in mice shows activation of the p35/Cdk5 kinase is required for neurite growth as mice deficient in p35 exhibited defects in cortical lamination and fasciculation of axon fibers (Nikolic M., et. al. 1996) (Chae T., et al. 1997).

In addition to Cdk5 acting as a mediator in neuronal survival, studies have shown the role of Cdk5 in mediating neuronal cell death. More specifically, a cleaved fragment of the Cdk5 activator (p35) by the Calpain enzyme (p25), releases active Cdk5 into the cytosol for prolonged activation to mediate neuronal cell death seen in various models displaying neurodegenerative diseases (Kusakawa G., et. al. 2000) (Tsai L.H., et. al. 1994). Unfortunately, a large portion of the global population is affected by various neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease. Studies have indicated that phosphorylated tau protein, a microtubule associated protein, is the main constituent of neurofibril tangles in the brains of Alzheimer's patients (Mandelkow E. M. and Mandelkow E. 1998). Alzheimer patient brains show an increased accumulation of p25, and as mentioned earlier, allows for prolonged activation of Cdk5 (Patrick G., et. al. 1999). When Cdk5 is deregulated through p25 accumulation, tau protein is phosphorylated and cannot effectively bind microtubules (Patrick G., et. al. 1999).

Cdk5 As a Regulator of Microtubule Proteins

Given the unique characteristics and functions of Cdk5, it is a great candidate to study for its role in maintaining healthy neuronal functions. More specifically, addressing how Cdk5 regulates healthy functioning neurons and what proteins Cdk5 is regulating can lead to a better understanding of healthy and diseased neurons. Previously published studies have shown Cdk5

directly influencing microtubule dynamics by phosphorylating Sirtuin-2 (SIRT-2), a histone deacetylase, to inhibit its activity to favor acetylation of α-tubulin (North BJ, et. al., 2003). When α-tubulin is acetylated, this promotes microtubule stability and growth in axons (North BJ, et. al., 2003). Additionally, Cdk5 phosphorylation activity has been shown to affect microtubule dynamics through microtubule associated proteins. More specifically, the phosphorylation of specific microtubule associated proteins by Cdk5 is necessary to promote the association between microtubules and the microtubule associated proteins such as collapsing response mediator protein 2 (CRMP2), Axin, stathmin, doublecortin, tubulin polymerization-promoting protein (TPPP), tau, and MAP1B. CRMP2 can either be inhibited or activated by phosphorylation of Cdk5 to promote either growth cone collapse or axonal elongation (Yamashita N, et. al., 2012). Phosphorylation of microtubule associated proteins by Cdk5 can either increase their affinity for binding microtubule, therefore promoting stability and polymerization, or can decrease their affinity for binding microtubules, therefore inhibiting polymerization and stability. For example, when MAP1B is phosphorylated by Cdk5, its affinity for microtubules is enhanced (Paglini G., et. al., 1998), whereas phosphorylation of doublecortin reduces its affinity for microtubules to inhibit microtubule polymerization and neuronal migration (Tanaka T., et. al., 2004). Furthermore, in vitro and in vivo experiments indicate that multi-site directed phosphorylation of TPPP results in decreased microtubule assembling activity (Hlavanda E., et. al., 2007). The reduction of microtubule affinity and stabilization following phosphorylation by Cdk5 is also seen with the microtubule associated protein, tau, which occurs during axonal growth but not during stabilization and synaptogenesis (Brion JP., et. al., 1994). An additional study to support Cdk5 regulation of microtubules shows that phosphorylation of stathmin at Ser38 releases tubulin bound to it to promote in axonal growth and branching

(Hayahi K., et. al., 2006). These studies have shown Cdk5 as a regulator in microtubule dynamics through phosphorylation of microtubule associated proteins mainly in the axon of neuronal cells. Given the significant role Cdk5 has on microtubule dynamics in the axons and the difference in microtubule polarity in the axons and dendrites, we want to study the regulation of microtubule dynamics by Cdk5 in the dendrites of neuronal cells.

Sub-Chapter 3: The Benefits of Using Class I ddAE *Drosophila* Neurons to Study Cytoskeletal Regulation

Numerous studies have shown Cdk5 playing a significant role in cytoskeletal dynamics in various model systems such as mice and *C. elegans* (Goodwin, P. R., et al. 2012) (Shah K., & Lahiri, D. K. 2016). Similar to humans, altered activity of Cdk5 in *Drosophila* leads to neurodegeneration in the adult flies (Mamlumbres M. 2014). This allows for the properties of Cdk5 to be applicably studied *Drosophila*. *Drosophila* make great model organisms due to their short life span (40 to 120 days), their ability to produce a large number of identical progeny, and their highly complex brain (Hirth F. 2010). As a result, *Drosophila* can easily be analyzed to study the structural and functional properties of their neurons (Hirth F. 2010). Additionally, neurons in *Drosophila* and mammals are very similar. This makes it easy to relate findings in *Drosophila to* mammalian species such as humans (Spindler SR and Hartenstein V. 2010). Furthermore, given the easily modified genome of *Drosophila,* proteins and mechanisms of interest can be analyzed by tagging them with fluorescent proteins or with the use of other genetic tools. Such genetic tools are utilized when exploring the function of Cdk5 in cells (Hirth F. 2010). Previous studies have used Drosophila to study microtubule dynamics in both the axons and dendrites. Our aim for understanding how Cdk5 regulates microtubule dynamics in

dendritic arborization neurons using the model organism *Drosophila* can allude to a greater understanding of healthy neurons as well as diseased state neurons. After our initial discovery—decreased localization of γ-tubulin to dendritic branch points of class I DA *Drosophila* neurons—we wondered the significance of Cdk5 to maintain microtubule dynamics within DA neurons. Based on our initial finding, we formed two alternative hypotheses for the specificity of Cdk5 in localizing γ-tubulin to dendritic branch points therefore regulating microtubule polarity: H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 4).

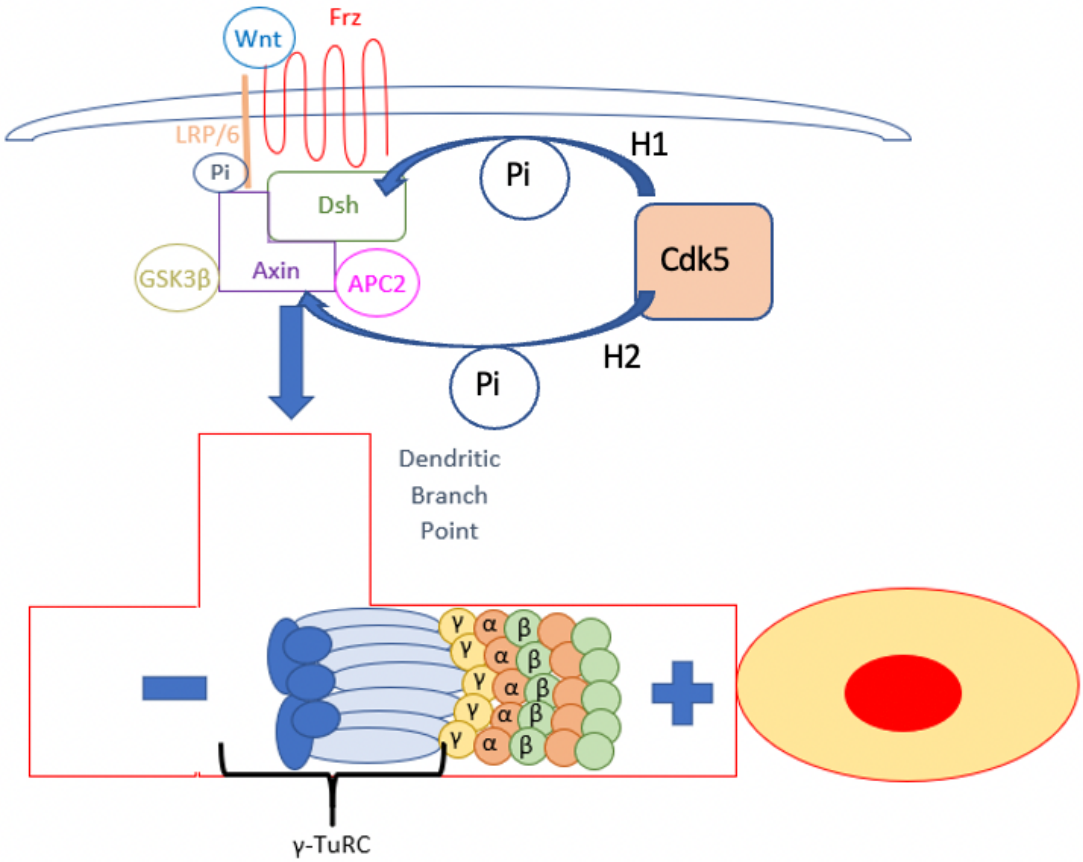

Figure 4. Two alternative hypotheses being addressed by our project. H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway. This hypothesis, represented by H1, shows phosphorylation activity occurring upstream of Axin (on Dsh) to regulate microtubule polarity within the dendritic branches. H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway. This hypothesis, represented by H2, displays phosphorylation activity occurring directly on Axin by Cdk5 to regulate microtubule polarity within dendritic branches.

Chapter 2

Materials & Methods

Sub-Chapter 1: Cloning Techniques

To clone Cdk5-GFP into a plasmid, the first step was to use SnapGene to determine an appropriate cloning vector. Specifically, we performed C-terminal infusion cloning experiment from the cDNA of the Cdk5 gene to insert the Cdk5 gene upstream of the GFP gene, which were both expressed under a UAS Gal4 driver. This cloning vector contained an antibiotic selectable marker (Ampicillin) as well as a GFP tag that would tack onto the Cdk5 CDS upon insertion. Restriction enzymes—EcoR1-HF and Spe1—were selected to excise the Cdk5 coding sequence, as well as to cleave the multiple cloning sites of my cloning vector for insertion. Following restriction digestion, digested Cdk5 CDS and cloning vector were run on an agarose gel to ensure that complete digestions were made and that the expected products were formed. After confirmation of digestion products, a ligation reaction was performed between the digested vector and Cdk5 CDS. The cloned vector was then transformed into competent E. Coli cells, and large numbers of the cloned vector were produced. E. coli that had taken up the plasmid were isolated via antibiotic selection. E. coli cells that were successfully transfected were then lysed, and the cloned vector was isolated. A sample of the isolated cloned vector was linearized—using one restriction enzyme—and run on an agarose gel to determine whether the desired plasmid was formed. After confirmation that the CDK5 CDS was cloned into the vector, we used polymerase chain reaction (PCR) to amplify and purity the sample. Then, the plasmid was sent off to be introduced into a Drosophila germ line.

Sub-Chapter 2: Confocal *In Vivo* Microscopy Imaging Techniques

To prepare larvae for *in vivo* microscopy, I mated virgin female flies with a tester line background (i.e. dicer2, mcd8-RFP; 221 Gal4, APC2-GFP/Tm6) to male flies with an RNAi background (i.e. CDK5 RNAi). Flies were mated in *Drosophila* bottles sealed with a removable cap containing *Drosophila* cornmeal media. Embryos were deposited on these caps and are collected at 24-hour intervals. Typically, up to 4 caps were collected for a single cross. Each collected cap was incubated at 25 degrees Celsius for 3 days, leading to the Development of live 3rd instar *Drosophila* larvae. To prepare 3rd instar larvae for microscopy, they were individually isolated from their respective cornmeal cap and dipped in water to remove any residual cornmeal media. Washed larvae were then gently placed ventral side down on a microscope slide that has a circular patch of dried agar at its center for visualization. Larvae were secured in this ventral down position by applying pressure via a microscope coverslip and securing with clear tape. Mounted larvae were imaged using the Zeiss LSM800 microscope. Larvae were located using 10x objective, and individual ddaE neurons in segments a2-a4 were located using 63x oil (NA1.42) (Zeiss) objective.

Sub-Chapter 3: *Drosophila* Stock Storage and Genetics

All genetically modified *Drosophila* stocks were obtained from the Bloomington *Drosophila* Center at Indiana University and the Vienna *Drosophila* Resource Center for our experimental analysis. Additional tester lines were created in the Roll's Lab using 2;3 *Drosophila* genetics and recombination techniques (Kaufman, T. C. 2017). A comprehensive list

of all tester, mutant, and RNAi fly lines are listed at the end of the materials and methods section. Flies were stored in plastic bottles and vials with cornmeal (yeast, soy flour, cornmeal, agar, corn syrup, propionic acid, and water) fly media at the bottom and the tops were closed using Styrofoam plugs and cotton balls, respectively. Stocks were placed in a humidity-controlled room at 25 °C for long-term storage and stocks being used to collect virgin female and male flies for genetic crosses were stored in 18 °C. All stocks were flipped into new bottles/vials every three to four weeks for sanitary purposes.

Sub-Chapter 4: Fluorescence Intensity Analysis of Data using Computer Software

For quantification of protein localization, I measured GFP fluorescence intensity (corresponding to GFP tagged proteins) at branch points and non-branch points of Class I ddaE neurons. The first step in obtaining these measurements was to obtain z-stack images of the dendrites of Class I ddaE neurons via the LSM800 microscope. These z-stack images were compiled into a single image for localization analysis by using FIJI to produce a max projection of all slices in the stack (Schindelin, J. et al. 2012). For GFP tagged proteins with diffuse localization at branch points, area within branch points as well as non-branch point regions of the dendrite arbor were manually outlined. Average pixel intensities of GFP within the outlined branch and non-branch regions were then measured via FIJI (Schindelin, J. et al. 2012). To normalize for background GFP pixel intensity, GFP pixel intensity at non-branch point regions of the dendrite arbor were subtracted from GFP pixel intensity values at branch points of the dendrite arbor. For GFP tagged proteins with tight, bright puncta at sites of localization, a binary quantification system was used. For example, a protein could be quantified as either being

present or absent at branch points. GFP intensity at 8-12 branch points and non-branch points are measured for every cell I analyzed.

Sub-Chapter 5: Microtubule Polarity Assay

To quantify microtubule polarity after carrying out CDK5 knockout, I captured 300 frame videos of EB1-GFP trafficking in the dendrites of Class I ddaE neurons using the LSM800 microscope. All 3rd instar larvae were alive during these video captures. All videos were 300 seconds long (1 frame per second). Videos of these Class I ddaE neurons were captured at 63x magnification using the 63x oil (NA1.4) (Zeiss) objective. EB1-GFP was visualized by exposing larvae to 470nm light. All movies were aligned and stabilized using FIJI prior to analysis of microtubule polarity (Schindelin, J. et al. 2012). For quantification, EB1-GFP comets were designated as growing either away from, or towards the neuronal soma and total number of comets moving in either direction was counted. I created kymographs representative of this data using FIJI (Schindelin, J. et al. 2012). Statistical analysis of data was conducted via logistical regression.

Sub-Chapter 6: *Drosophila* Stocks

RNAi and Mutant list

For all of the fluorescence microscopy localization experiments the control RNAi line was Reticulin 2 RNAi (RTNL2 RNAi 33320) CG identification: CG1279 found on chromosome II. One of the experimental Cyclin Dependent Kinase 5 (Cdk5) RNAi lines, was used for the

majority of the localization experiments. This line was Cyclin Dependent Kinase 5 (Cdk5) RNAi 35855 CG identification: CG8203 found on chromosome II. The other experimental RNAi line was Cyclin Dependent Kinase 5 (Cdk5) RNAi line (Cdk5 RNAi 104491) CG identification: CG8203 found on chromosome II.

Fly Strains, Tagged Constructs, and Antibodies

For our localization experiments, we used *Drosophila* stock lines which contained fluorescently tagged proteins of interest. All lines were expressed under the UAS Gal4 system. The following lines were used to collect virgin females to cross with males of the RNAi lines: Dicer 2, MCD8-RFP; 221 Gal4, APC2-GFP/TM6, Dicer 2, MCD8-RFP; 221 Gal4, Axin-GFP/TM6, Dicer 2, MCD8-RFP; 221 Gal4, Dsh-GFP/TM6, Dicer 2, MCD8-RFP; 221 Gal4, EB1-GFP/TM6, Dicer 2, MCD8-RFP; 221 Gal4, γ-tub-GFP/TM6, Dicer 2, mCherry-RFP; 221 Gal4, Fz2-GFP/TM6. Additionally, we examined the localization of Cdk5 using similar microscopy techniques, but we did not cross this line to a mutant RNAi line. The line used for Cdk5 localization was Dicer 2, MCD8-RFP; 221 Gal4, Cdk5-GFP/TM6.

Chapter 3

Results

Sub-Chapter 1: Analysis of γ-tubulin Localization to Dendritic Branch Points and Microtubule Polarity when Cdk5 is Depleted in the Cell

Given the importance of Cdk5 in maintaining healthy neuronal function (Figure 3) (Shah K. & Lahiri, D. K. 2016) and the role that dysregulation of Cdk5 plays in the Development of neurodegenerative diseases (Malumbres M. 2014), we set out to discover the specific role of Cdk5 in DA neurons using the model organism *Drosophila*. We also know the importance of microtubules ability to maintain healthy neuronal function through trafficking proteins throughout the cell with a distinct polarity (Van de Willige, D. et. al., 2016). Additionally, γ-tubulin, a component of the γ-tubulin ring complex (γ-TuRC), is important for M.T. nucleation processes and loss of γ-tubulin of γ-TuRC factors results in reduced M.T. polymerization (Delandre C., Amikura R., and Moore A. 2016). Studying the Cdk5's role in maintaining M.T. dynamics is a key experiment to understanding the role of Cdk5 in DA neurons. We hypothesized that Cdk5 is regulating microtubule polarity by either activating Axin through a parallel pathway or by directly activating proteins within the Wnt signaling pathway to localize γ-tubulin to dendritic branch points (Figure 4).

In order to address the central question about Cdk5's specific role in Class I DA neurons of *Drosophila*, I first set up an experiment to study the effects of γ-tubulin localization when Cdk5 is knockdown. Our hypothesis states Cdk5 is essential for regulating microtubule dynamics

by directly regulating proteins within the Wnt signaling pathway. If Cdk5 plays a significant role in localizing y-tubulin to the branch points of Class I DA *Drosophila* neurons, we would expect to see a decrease in the localization of γ-tubulin to the branch points when Cdk5 is depleted in the cell via RNAi methodology. Given γ-tubulin role in maintaining minus-end out microtubule polarity in mature *Drosophila* dendrites (Yau, K. W., et al. 2014), if Cdk5 leads to decreased localization of γ-tubulin to branch points, this would suggest that Cdk5 plays an important role in maintaining proper microtubule polarity for healthy neuronal function. This experiment was performed by crossing virgin female *Drosophila* with green fluorescence marker for the protein γ-tubulin (γ-Tub GFP) with male *Drosophila* of either a control RNAi line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS γ-tubulin-GFP)/TM6 x RTNL2 RNAi 33320) or the Cdk5 RNAi line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS γ-tubulin-GFP)/TM6 x Cdk5 RNAi 35855). γ-Tubulin GFP has been used in previous experiments. For example, in the paper "Centrosome-independent mitotic spindle formation in vertebrates" constitutive expression of γ-tubulin GFP was used to define the boundaries of the centrosome in a mammalian cell line (Khodjakov, A. et al., 2000). Then, 3rd instar larvae were subjected to fluorescence microscopy for imaging for each cross (Figure 5A and 5B). 18 cells were imaged for the control cross, and 13 cells were imaged for the experimental Cdk5 RNAi cross.

After imaging, the computer software, FIJI (Schindelin, J. et al. 2012), was used to quantitate γ-tubulin to Class I dendritic branch points by normalizing the amount of GFP at each branch point to the GFP values at non-branch points. The fluorescence intensity was measured at the dendritic branch points and the non-branch points. Then the subtraction of the non-branch points fluorescence intensity from the branch point fluorescence intensity calculations gave the γ-tubulin localization value at each branch point without background (between branch points or

in the background of the image)—this was designated as "normalized" γ-tubulin localization. Each image, control RNAi and for Cdk5 RNAi, was used to calculate a normalized γ-tubulin GFP intensity value at branch points. The overall average normalized value of γ-tubulin-GFP to branch points was used to graphically display the difference between the GFP intensity at dendritic branch points for the control RNAi cross and the Cdk5 RNAi cross (Figure 6). Then, the average normalized fluorescence intensity due to γ-tubulin-GFP at dendritic branch points is plotted on the y-axis with the control RNAi and Cdk5 RNAi plotted on the x axis (Figure 6).

Analysis of our results for γ-tubulin localization experiment indicate significant results. Figures 6A and 6B serve as representative example images of the overall distribution of γ-tubulin to dendritic branch points in both control cells and Cdk5 RNAi cells. The distribution of γ-tubulin appears significantly less in the Cdk5 mutant organisms. Additionally, quantitative analysis of these cells using the images captured from fluorescence microscopy, provided evidence the qualitative observation—Cdk5 mutant organisms displayed a decrease in γ-tubulin localization to dendritic branch points. With a calculated p-value of 0.006 at the 0.05 alpha level, the results were statistically significant. As a result, it appears Cdk5 functions in regulating γ-tubulin localization to branch points of class I DA neurons in *Drosophila*.

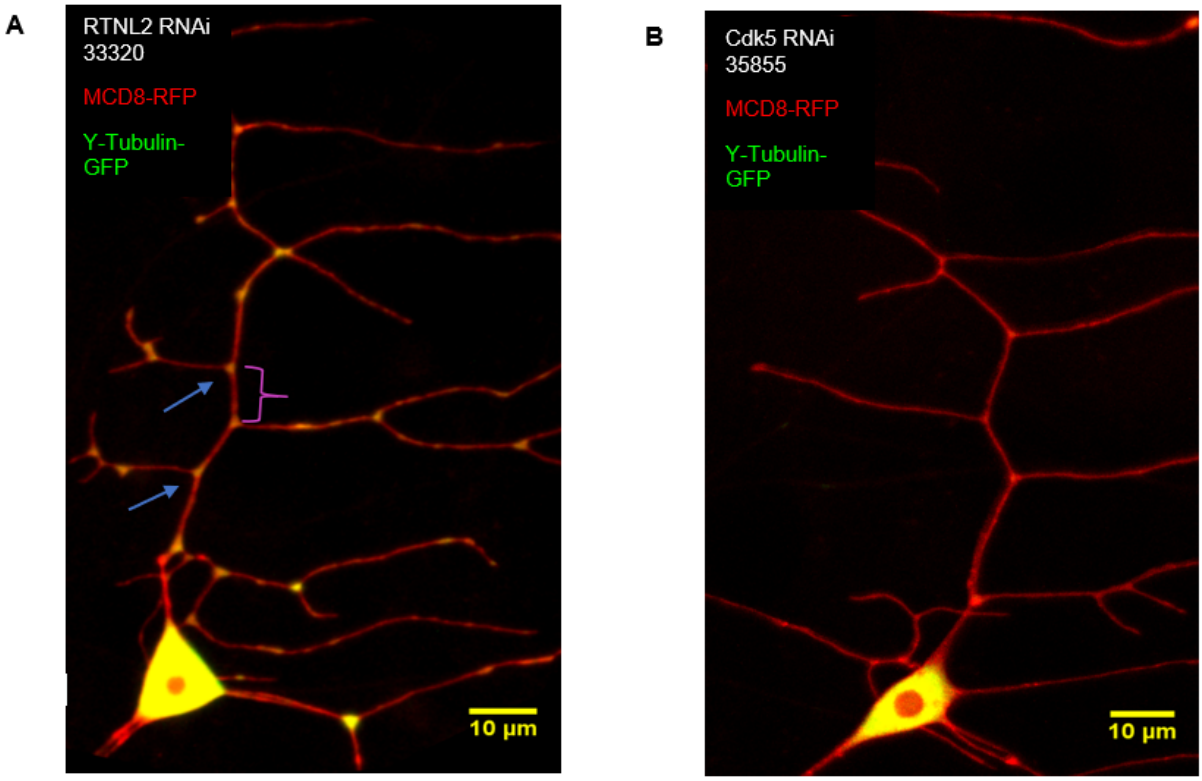

Figure 5. Images of γ-tubulin-GFP localization to dendritic branch points with either (A) control RNAi or (B) Cdk5 RNAi in Class I DA Drosophila neurons. (A) Example image of the control line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS γ-tubulin-GFP)/TM6 x RTNL2 RNAi 33320). (B) Example image of the knockdown Cdk5 (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS γ-tubulin-GFP)/TM6 x Cdk5 RNAi 35855). (A) Blue arrows represent branch points and purple right brace symbol shows the non-branch points (spaces between adjacent branch points).

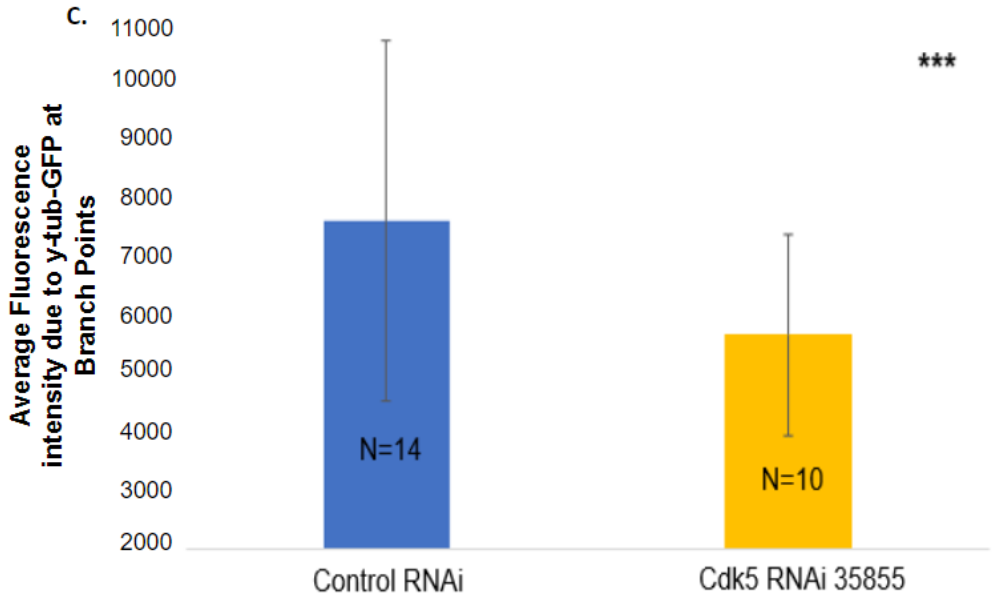

Figure 6. Average normalized γ-tubulin GFP intensity at dendritic branch points of Class I DA neurons in Drosophila for a control RNAi (N=14) and Cdk5 RNAi (N=10). γ-tubulin localization was quantified by analyzing the fluorescence intensity of the neurons. The fluorescence intensity was measured at the dendritic branch points and the non-branch points. Then the subtraction of the non-branch points fluorescence intensity from the branch point fluorescence intensity calculations gave the normalized γ-tubulin localization value. Distribution of the data obtained from the knockdown of Cdk5 35855. A t-test was conducted to obtain a p value of <0.001.

Next, we wanted to study microtubule polarity in these cells when Cdk5 levels are depleted, as we confirmed from our experiments and previous experiments, γ-tubulin is a key protein that regulates nucleation and polarity of microtubules. To analyze microtubule dynamics in cells, we crossed virgin female *Drosophila* with green fluorescently tagged EB1 proteins (EB1 binds to polymerizing/plus ends of microtubules) with either a male *Drosophila* of a control RNAi line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS EB1-GFP)/TM6 x RTNL2 RNAi 33320) or the Cdk5 RNAi line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS EB1-GFP)/TM6 x Cdk5 RNAi 35855). From the crosses, we took 300 second videos of 3rd instar larvae dendritic branch points to analyze microtubule polarity within the dendrites. The videos

could be used to create kymograph images that display the microtubules in both time and space (Figure 7). Moving along the kymograph displays the microtubules at different time points. Normal microtubule kymographs show microtubules moving down and to the right (Figure 7A blue lines). If the microtubule polarity is mixed, there will be microtubules moving in directions other than down and to the right of the kymograph (Figure 7B red lines). Specifically, the kymographs indicate microtubules traveling in both directions (Red and Blue lines) for the Cdk5 knockdown cells and uniform microtubules polarity in the control RNAi (Blue lines).

After generating our kymograph images, we determined the percentage of microtubules with plus ends polymerizing away from the cell body for each cross, control RNAi and Cdk5 RNAi. In order to calculate this percentage, we counted the microtubule comets migrating away from the cell body in the dendrites compared to the number of comets throughout the dendrites to determine the percent of microtubules with plus ends polymerizing away from the cell body (Figure 8). Displayed in Figure 8, the percent of microtubules with plus-end oriented away from the cell body is plotted on the y axis and the control RNAi and Cdk5 RNAi are plotted on the x axis. For the control RNAi and Cdk5 RNAi 160 and 80 microtubule samples were analyzed, respectively. The p-value for plus-end out microtubule dynamics between the control and Cdk5 RNAi lines using a categorical Chi-squared test, was 0.00515 at the 0.05 alpha level – suggesting a significantly greater number of microtubules with mixed polarity in Cdk5 RNAi cells. Additionally, we calculated the comets normalized—microtubules nucleating from the branch point—for each cross, control RNAi and Cdk5 RNAi (Figure 9). Comets were normalized by calculating the ratio of the number of comets to the length of the main dendritic branch. Figure 8 displays the comets normalized on the y axis and the control RNAi and Cdk5 RNAi plotted on the x axis. For the control RNAi and Cdk5 RNAi 19 and 15 samples were analyzed, respectively. The

p-value, using a two-mean t-test to determine the statistical significance of the data, was 0.006 at the 0.05 alpha level – suggesting a significant decrease in comets observed in Cdk5 RNAi cells. Based on these results, Cdk5 is implicated in regulating microtubule polarity in class I DA *Drosophila* neurons.

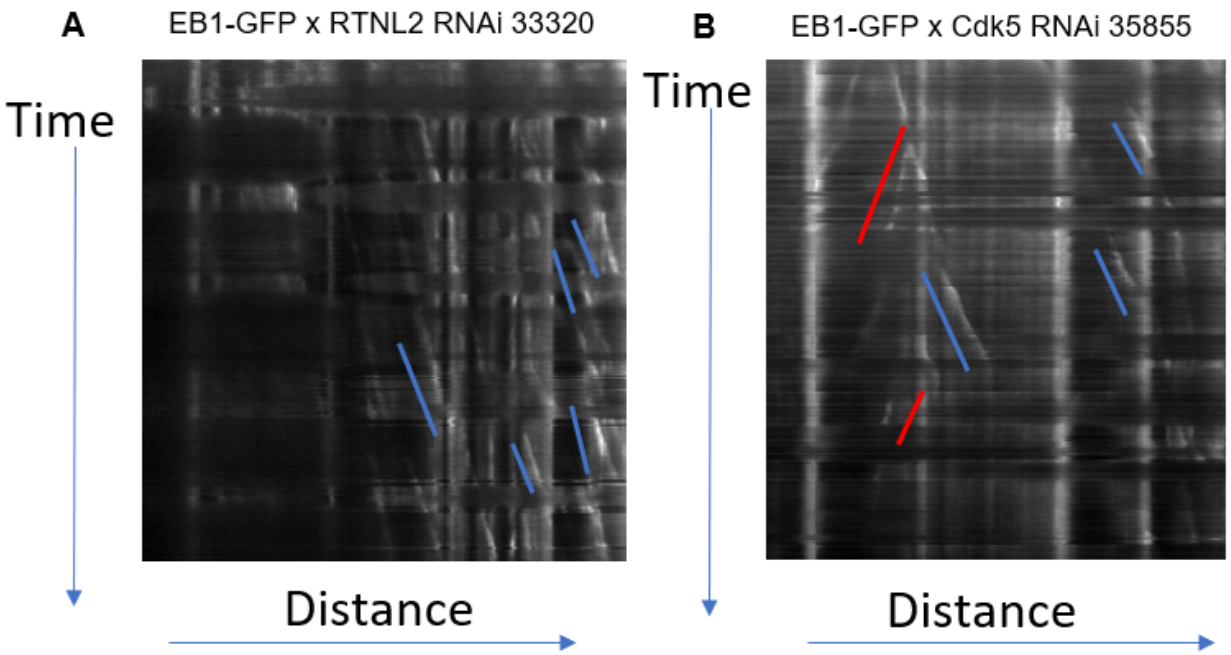

Figure 7. Kymograph representation of microtubule polarity in Time vs Distance. (A) is a kymograph of the control cross (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAs EB1-GFP)/TM6 x RTNL2 RNAi 33320). (B) is a kymograph of the Cdk5 RNAi cross (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS EB1-GFP)/TM6 x Cdk5 RNAi 35855). Blue lines indicate normal movement of microtubules and red arrows indicate abnormal movement of microtubules.

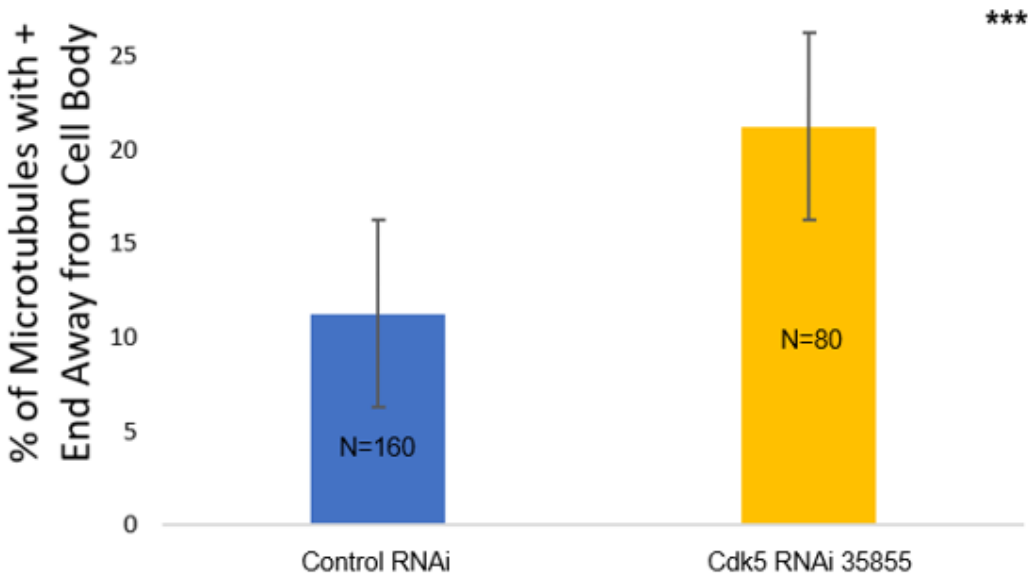

Figure 8. Percent of microtubules with the plus end polymerizing away from the cell body. Figure represents the percentage of microtubules polarizing plus end out (away from the cell body). The control cross (UAS Dicer 2, UAS Mcd8-RFP; (UAS 221 Gal 4, UAs EB1-GFP)/TM6 x RTNL2 RNAi 33320) (N=160) and the Cdk5 RNAi cross (UAS Dicer 2, UAS Mcd8-RFP; (UAS 221 Gal 4, UAS EB1-GFP)/TM6 x Cdk5 RNAi 35855) (N=80). A categorical Chi-squared test was used to calculate a p value of 0.00515.

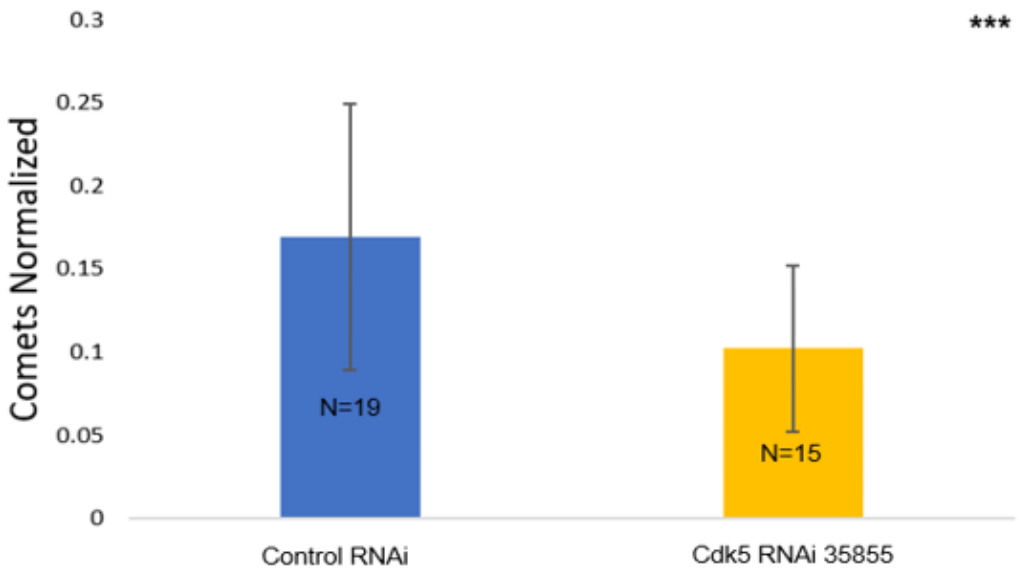

Figure 9. The comets normalized in Class I DA Drosophila neurons. Figure depicts the average number of normalized comets (number of comets/the total length of the main dendrite) localized within the cell of control RNAi flies (N=19) and Cdk5 RNAi flies (N=15). The p-value, using a two-mean t-test to determine the statistical significance of the data, was 0.006 at the 0.05 alpha level.

Sub-Chapter 2: Localization assay of mitochondria to dendritic branch points when Cdk5 is depleted in the cell

In a prior study, mitochondria were visualized to localize to dendritic branch points using GFP tagged mitochondria (Hill S.E., et. al., 2012). Additionally, the localization of mitochondria to dendritic branch points occurs early during development, prior to microtubules obtaining a uniform polarity in the cell (Hill S.E., et. al., 2012). Given this, we questioned whether Cdk5 played a general or specific role in γ-tubulin localization. More specifically, does Cdk5 only regulate γ-tubulin localization, or does it have other cellular functions – such as regulation of mitochondrial localization. In order to address this question, we designed a localization experiment measuring mitochondrial localization to dendritic branch points. This localization

experiment can allude to the specificity of Cdk5 for γ-tubulin localization to dendritic branch points because mitochondria is not a protein within the Wnt signaling pathway. If the localization of mitochondria is significantly different in Cdk5 RNAi organisms when compared to the control RNAi organisms, this could indicate a more general role of Cdk5 in these cells, possibly through the minus-end directed motor protein, dynein. Whereas, if the localization of mitochondria is unaffected by the knockdown of Cdk5, this could indicate a more specific role of Cdk5 for localizing γ-tubulin to dendritic branch points. To support our hypothesis, Cdk5 is directly acting on proteins within the Wnt signaling pathway to regulate microtubule dynamics in Class I DA neurons, mitochondrial localization should not change between the control organisms and the Cdk5 knockdown organisms. In this microscopy experiment, a control RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x RTNL2 RNAi 33320) was used for comparison with the experimental Cdk5 RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x Cdk5 RNAi 35855). For our mitochondrial localization experiment, virgin female *Drosophila* with a green fluorescence marker for the protein mitochondria (Mito-GFP) with male *Drosophila* of either a control RNAi line or the Cdk5 RNAi line. Then, 3rd instar larvae were subjected to fluorescence microscopy (Figure 10A and 10B). From these images, we concluded depleting Cdk5 in the class I DA *Drosophila* neurons does not cause an obvious difference in localization of mitochondria to dendritic branch points.

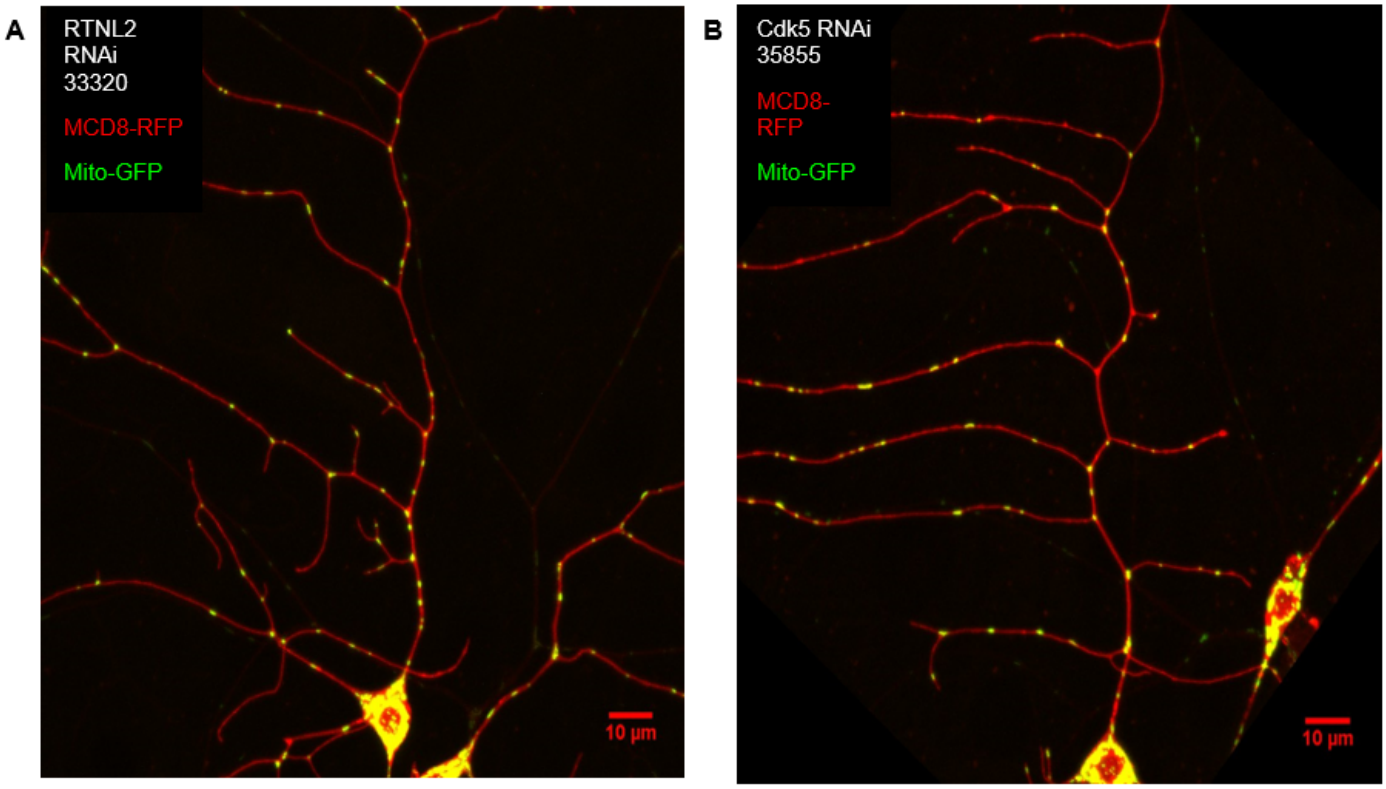

Figure 10. (A) The control cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x RTNL2 RNAi 33320), displays an example image of Apc2 localization to the branch points of Class I DA neurons. (B) The experimental cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x Cdk5 RNAi 35855), displays an image from the Cdk5 knockdown experiment.

In order to quantitatively analyze mitochondrial localization, we measured the average percent of dendritic branches with mitochondria under both control and Cdk5 RNAi conditions. To calculate the percent of mitochondria localizing to the dendritic branches we used the formula, $\frac{\%\ of\ branch\ points\ with\ Mito-GFP}{Total\ number\ of\ branch\ points}\ X\ 100$, for each control RNAi image (N=14) and each Cdk5 RNAi image (N=14). Following this calculation, we averaged the percent of mitochondria at the dendritic branch points for the control RNAi (81.3%) and for the Cdk5 RNAi (85.1%). Finally, each average percent and standard deviation (control RNAi: 18.3% and Cdk5 RNAi:

12.7%) was subjected to a two-mean t-test to determine the statistical significance of the data. The p-value was 0.527, at the 0.05 alpha level, suggesting that Cdk5 does not regulate mitochondrial localization in the dendrites of class I DA *Drosophila* neurons. The average percent of dendritic branch points with mitochondria for each experimental cross, and their corresponding standard deviations (error bars), are displayed below (Figure 11A).

Additionally, we subjected our mitochondrial localization experiment to another quantitative analysis. We measured the total number of mitochondria in dendrites for the control RNAi and the Cdk5 RNAi organisms and averaged the total mitochondria, 51.5 and 51.1, respectively. We also calculated the standard deviations for the control RNAi and the Cdk5 RNAi to be 12.0 and 13.4, respectively. The average number of total mitochondria localizing to dendrites for the control RNAi and the Cdk5 RNAi values were subjected to a two-mean t-test to determine the statistical significance. The calculated p-value was 0.93, at the 0.05 alpha level, providing additional evidence that Cdk5 is not involved in regulation of mitochondrial localization. The average total number of mitochondria in the dendrites for each experimental cross, and their corresponding standard deviations (error bars), are displayed below (Figure 11B).

In addition to our quantitative analysis, qualitative analysis of our Mito-GFP with either the control RNAi or the Cdk5 RNAi did not show any significant changes in mitochondrial localization upon Cdk5 depletion (Figure 10A and 10B). Overall, the localization of mitochondria to dendritic branch points appears unchanged following Cdk5 knockdown in class I DA *Drosophila* neurons.

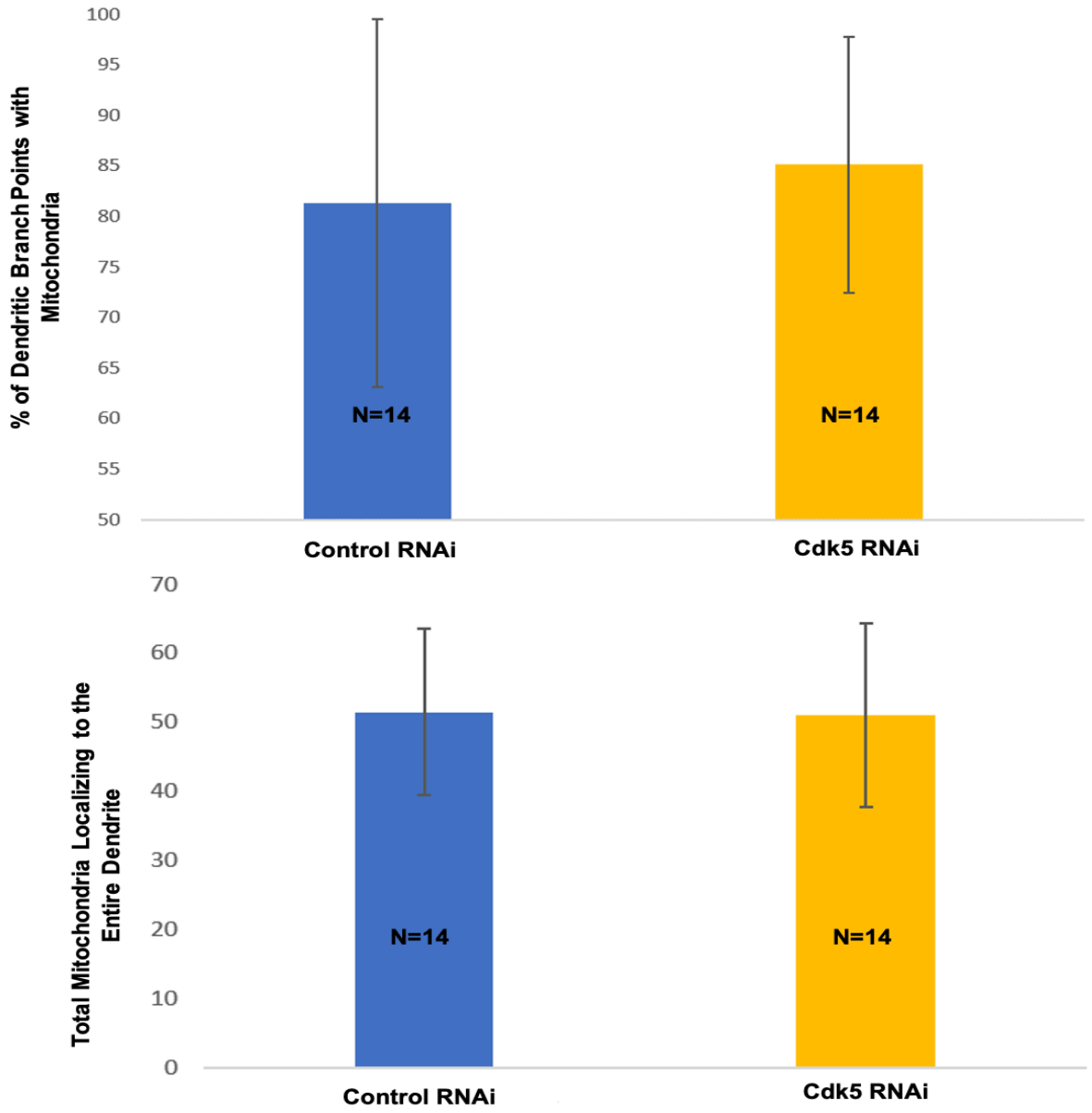

Figure 11. (A) Average percentage and standard error bars of dendritic branch points localizing Mitochondrion. The percent localized was quantified by (% of branch points with Mito-GFP)/(Total number of branch points) X 100. The percent of dendritic branch points with mitochondria is plotted against the control RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x RTNL2 RNAi 33320) and the Cdk5 RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x Cdk5 RNAi 35855). A two-mean t-test compared the control to the Cdk5 knockdown. The p-value was 0.527. (B) Average number of mitochondrion localizing to the whole dendrites and standard error bars of the control cross; (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x RTNL2 RNAi 33320) and the experimental cross; (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Mito-GFP)/TM6 x Cdk5 RNAi 35855). Calculations were computed by counting the total number of GFP tagged mitochondria in the dendrites and obtaining averages for each experiment. Following a two-mean t-test, the respective p-value was 0.93.

Sub-Chapter 3: Localization assay of APC2 to dendritic branch points when Cdk5 is depleted in the cell

Previously, Apc2 was found to localize to dendritic branch points in *Drosophila* neurons prior to assembly of uniform microtubule polarity, visualized using a GFP tagged Apc2 (Hill S.E., et. al., 2012). Additionally, Apc2 negatively regulates Wnt signaling in *Drosophila* embryos (McCartney, B.M., & Nathke, I.S. 2008) which makes it a unique candidate for studying its localization following knockdown of Cdk5 in the cells. Based on this discovery, we designed an experiment to test Apc2 localization when Cdk5 is knocked down in the cell. By testing Apc2 localization when Cdk5 levels are depleted in the cell, we could gain a better understanding about the role Cdk5 has in organizing microtubules at dendritic branch points. Whether Apc2 levels are affected by depleted Cdk5 levels in the cell cannot conclude or disprove our hypothesis since Apc2 is within the Wnt signaling pathway. If Apc2 levels are significantly different after analysis, this could mean Cdk5 has a specific role on localizing Apc2 to dendritic branch points to regulate microtubule dynamics. If Apc2 levels are not significantly different when Cdk5 is knocked down in the cell, this could indicate Cdk5 is responsible for activating another protein within the Wnt signaling pathway to regulate microtubule dynamics, but not Apc2. To conduct this localization experiment, we mated virgin female *Drosophila* with a GFP tagged Apc2 protein (Apc2-GFP) with the control RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x RTNL2 RNAi 33320) or the experimental Cdk5 RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x Cdk5 RNAi 35855) flies. Then, 3rd instar larvae were subjected to fluorescence microscopy (Figure 12A and

12B). 12 cells were imaged for the control cross, and 20 cells were imaged for the experimental Cdk5 RNAi cross.

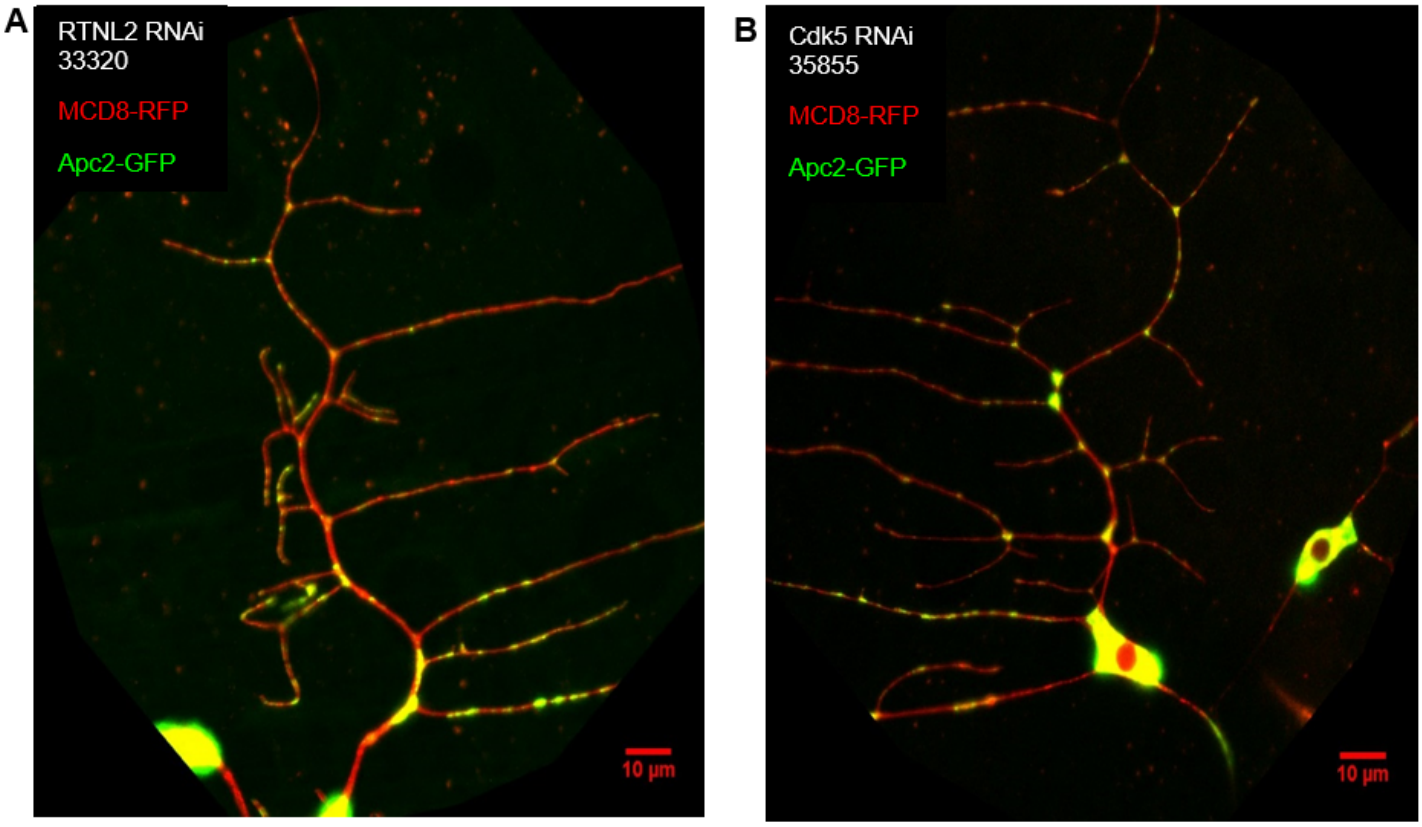

Figure 12. (A) The control cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x RTNL2 RNAi 33320), displays an example image of Apc2 localization to the branch points of Class I DA neurons. (B) The experimental cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Apc2-GFP)/TM6 x Cdk5 RNAi 35855), displays an image from the Cdk5 knockdown experiment.

We performed quantitative measurements of both the control RNAi and Cdk5 RNAi Apc2 localization experiments to determine the statistical significance of Apc2 localization to the dendritic branch points. We measured the average percent of dendritic branches with Apc2. To calculate the percent of Apc2 localizing to the dendritic branches we used the formula, $\frac{\%\ of\ branch\ points\ with\ Apc2-GFP}{Total\ number\ of\ branch\ points}\ X\ 100$, for each control RNAi image (n=9) and each Cdk5 RNAi image (n=15). Following this calculation, we averaged the percent of Apc2 at the dendritic

branch points for the control RNAi (89.7%) and for the Cdk5 RNAi (87.9%). Finally, each average percent and standard deviation (control RNAi: 9.9% and Cdk5 RNAi: 11.3%) was subjected to a two-mean t-test to determine the statistical significance of the data. The p-value was 0.698 at the 0.05 alpha level – suggesting that Cdk5 does not significantly regulate the localization of APC2 to dendritic branch points in class I DA *Drosophila* neurons. The average percent of dendritic branch point with APC2 for each experimental cross, and their corresponding standard deviations (error bars) are displayed below (Figure 13).

We also used the images from our APC2 localization (APC2-GFP) experiment for qualitative analysis (Figure 12A and 12B). Based on the images, we were unconvinced of a difference in the APC2 localization to dendritic branches. This provides supporting evidence that Cdk5 is not involved in regulating the localization of APC2 in the dendrites of class I DA neurons.

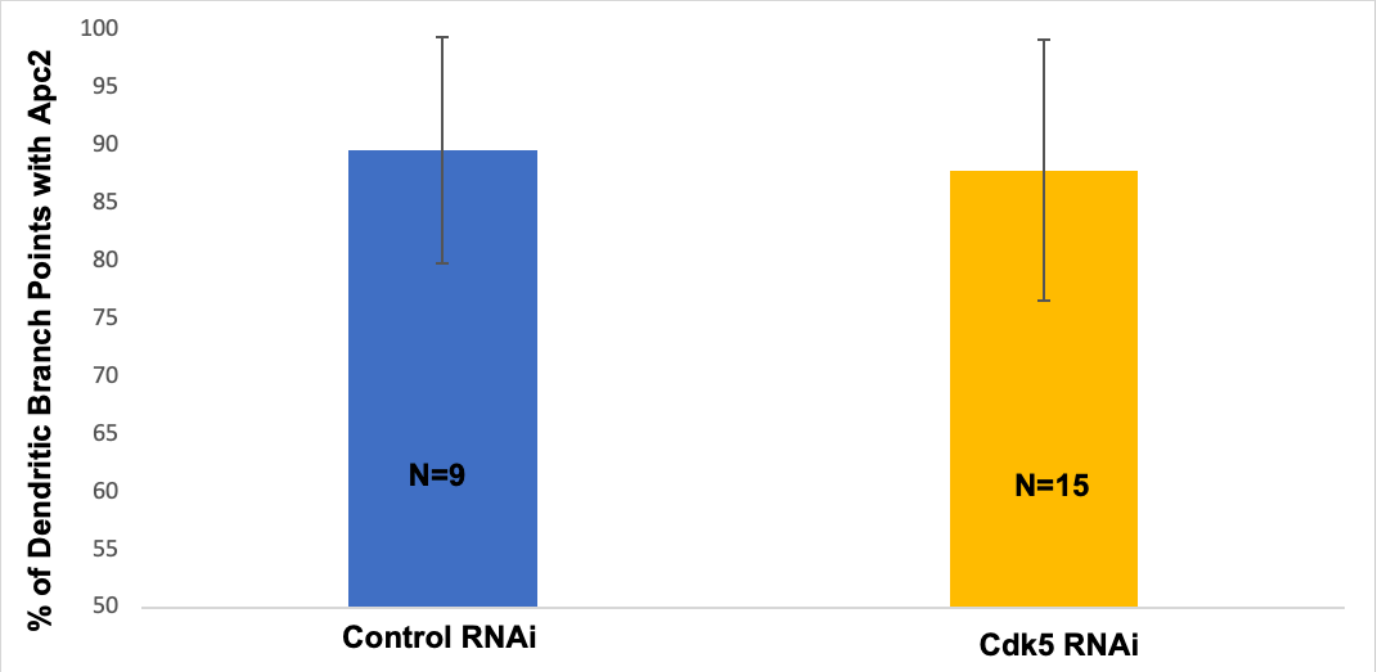

Figure 13. Average percentage and error bars of dendritic branch points localizing Apc2 for the control RNAi and Cdk5 RNAi experiments. The percent localized was quantified by (% of branch points with Apc2-GFP)/(Total number of branch points) X 100. A two-mean t-test was conducted resulting in a p value of 0.698 at the 0.05 alpha level.

Sub-Chapter 4: Localization assays of Wnt proteins when Cdk5 is depleted in the cell

As mentioned previously, proteins constituting the Wnt Signaling Pathway such as Axin, Disheveled (Dsh), and Frizzled (Fz) are necessary to localize γ-Tubulin to dendritic branch points (Weiner A.T, et. al. 2020). We aimed to study the localization patterns of each protein independently when Cdk5 levels were depleted in the cells. By conducting this experiment, we could gain a better understanding as to how Cdk5 might be regulating microtubule polarity in the cell. These experiments address our hypotheses and allow us to determine which hypothesis is most likely correct (Figure 5). First, we analyzed the localization patterns of Axin to dendritic branch points when Cdk5 levels were depleted in the cell. To conduct this experiment, virgin female *Drosophila* with green fluorescence marker for the protein Axin (Axin-GFP) with male *Drosophila* of either a control line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Axin-GFP)/TM6 x RTNL2 RNAi 33320) or the Cdk5 RNAi line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Axin-GFP)/TM6 x Cdk5 RNAi 35855). Then, 3rd instar larvae were subjected to fluorescence microscopy for imaging for each cross (Figure 14A and 14B). 20 cells were imaged for the control cross, and 21 cells were imaged for the experimental Cdk5 RNAi cross.

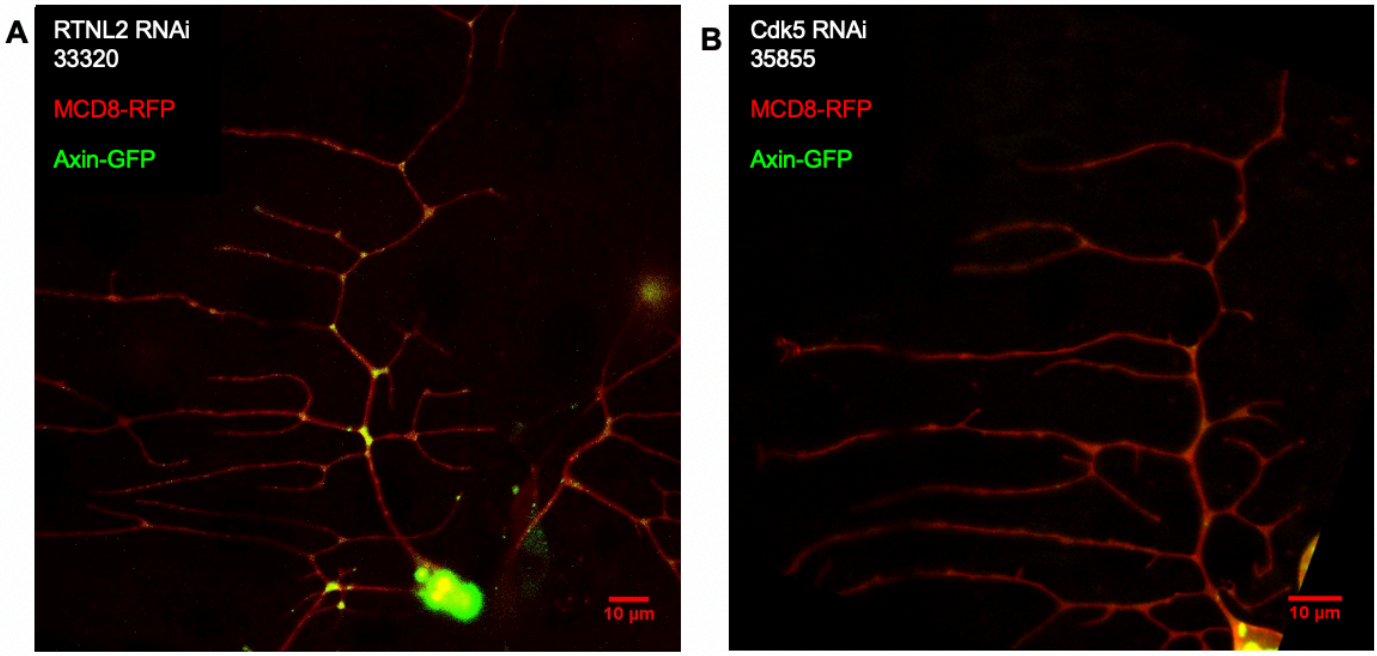

Figure 14. Axin localization to branch points of class I DA neurons in Drosophila following the knockdown of Cdk5 protein. (A) Example image of the control line (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Axin-GFP)/TM6 x RTNL2 RNAi 33320). (B) Example image of the knockdown of Cdk5 (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Axin-GFP)/TM6 x Cdk5 RNAi 35855).

Following fluorescence microscopy, the images were analyzed using the computer software, FIJI (Schindelin, J. et al. 2012). This allowed us to quantitate the Axin localization to class I dendritic branch points by measuring the amount of GFP at each branch point. To do so, the fluorescence intensity of Axin-GFP was measured at the dendritic branch points and the non-branch points. Then, the subtraction of the non-branch points fluorescence intensity from the branch point fluorescence intensity calculations gave the normalized Axin localization value (again, normalization constitutes subtraction of non-branch GFP intensity from branchpoint GFP intensity). The overall average normalized value of Axin-GFP to branch points for all images collected was used to graphically display the difference between the GFP intensity at dendritic branch points for the control RNAi cross and the Cdk5 RNAi cross (Figure 15). As depicted in

Figure 15, the average normalized fluorescence intensity due to Axin-GFP at dendritic branch points is plotted on the y-axis with the control RNAi and Cdk5 RNAi on the x axis. The number of animals for the control RNAi and Cdk5 RNAi were 18 and 20, respectively. Then, subjecting the data to a two-mean t-test determined the statistical significance of the data from the Axin localization experiment. The calculated p-value was less than 0.021 at the 0.05 alpha level – suggesting a significant decrease in Axin localization to branchpoints upon Cdk5 depletion.

To support our quantitative analysis, we examined our images of class I DA neuronal dendrites containing Axin-GFP under both control and Cdk5 RNAi conditions for qualitative analysis. Qualitative analysis revealed a visible decrease in localization of Axin to dendritic branch points in Cdk5 depleted cells.

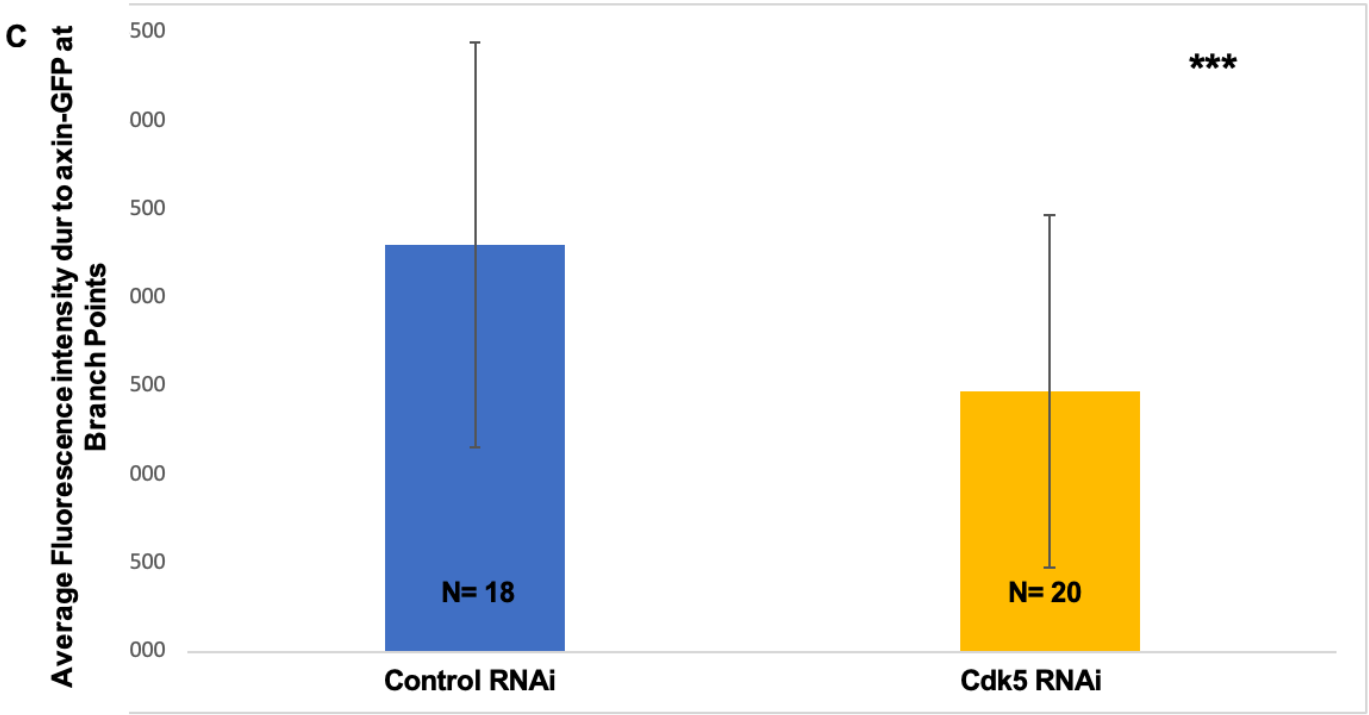

Figure 15. Axin localization was quantified by analyzing the fluorescence intensity of the neurons. The branch points were measured and the subtraction of the non-branch point from the branch point calculations to give the normalized Axin localization quantification. Distribution of the data obtained from the knockdown of Cdk5. A two-mean t-test was conducted with a p-value of 0.021 indicating a significant decrease in Axin localizing to branch points with the knockdown of Cdk5 35855.

After finding a significant decrease in Axin localization to Cdk5 knockdown cells, we aimed to study the next upstream protein in the Wnt pathway, Dsh, in cells with Cdk5 knocked down in the cell. By performing this experiment, we addressed our hypotheses addressing how Cdk5 may be regulating microtubule polarity in dendritic arborization neurons (Figure 4). To conduct this experiment, we crossed female *Drosophila* with green fluorescence marker for the protein Dsh (Dsh-GFP) with male *Drosophila* of one of the following line: control RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Dsh-GFP)/TM6 x RTNL2 RNAi 33320), Cdk5 RNAi 35855 (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Dsh-GFP)/TM6 x Cdk5 RNAi 35855), or Cdk5 104491 (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Dsh-GFP)/TM6 x Cdk5 RNAi 104491). We used two different Cdk5 RNAi lines to ensure the RNAi lines were knocking down Cdk5 in the cell. Then, 3rd instar larvae were subjected to fluorescence microscopy for imaging for each cross (Figure 16A, 16B, and 16C). For the control cross, Cdk5 RNAi 35855, and Cdk5 RNAi 104491 crosses, the number of cells imaged were 12, 24, and 20, respectively.

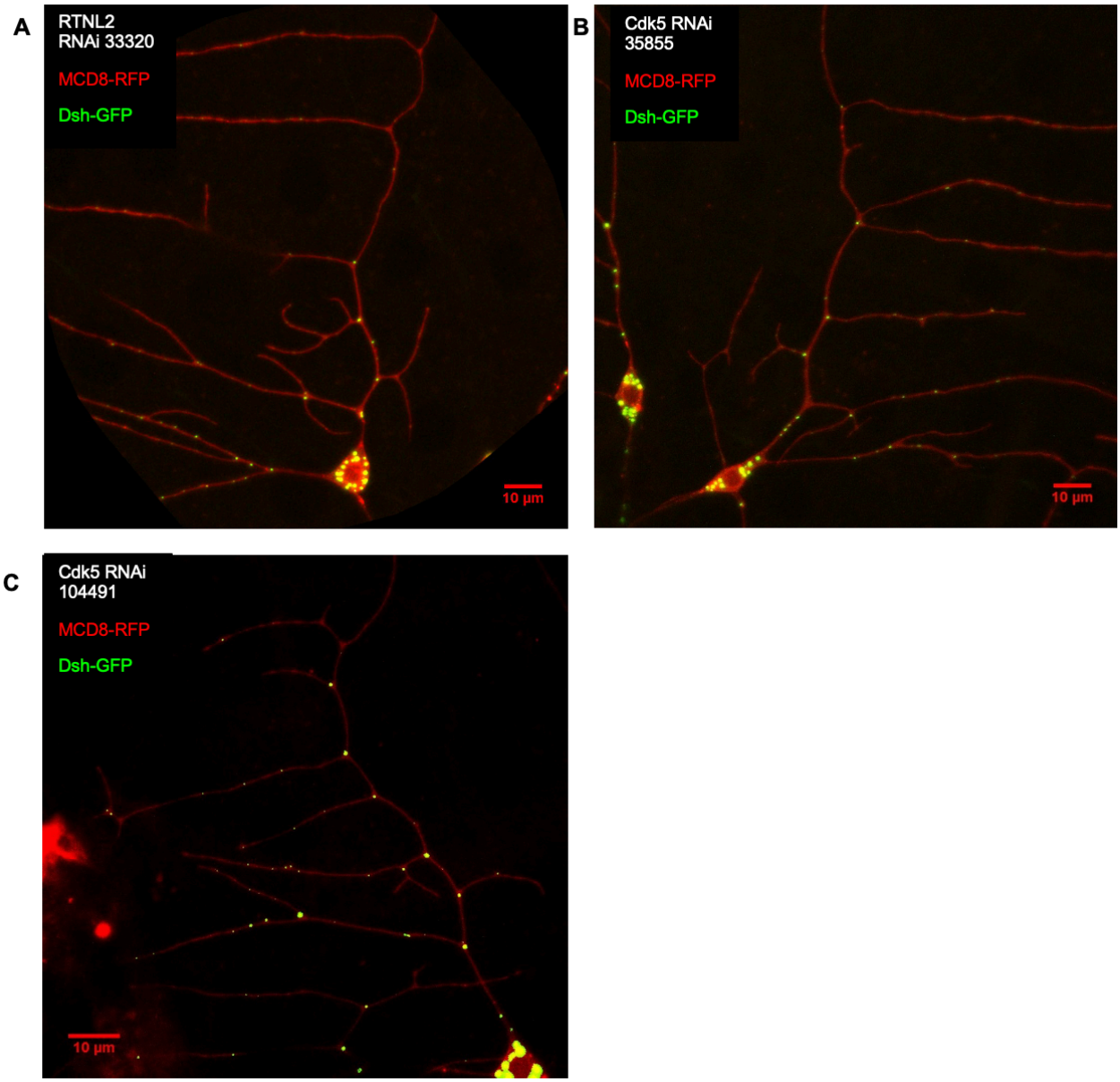

Figure 16. (A) The control cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Dsh-GFP)/TM6 x RTNL2 RNAi 33320), displays an example image of Dsh localization to the branch points of class I DA neurons. (B) The experimental cross, (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Dsh-GFP)/TM6 x Cdk5 RNAi 35855), displays an image from the Cdk5 knockdown experiment. (C) An experimental cross with a different Cdk5 line (UAS

Additionally, we performed quantitative measurements of all crosses: the control RNAi, Cdk5 RNAi 35855, and Cdk5 RNAi 104491. We measured the average percent of dendritic

branches with Dsh-GFP. To calculate the percent of Dsh localizing to the dendritic branches we used the formula, $\frac{\%\ of\ branch\ points\ with\ Apc2-GFP}{Total\ number\ of\ branch\ points}\ X\ 100$, for the control RNAi images (N=12) the Cdk5 RNAi 35855 images (N=24), and Cdk5 RNAi 104491 (N=20). Following this calculation, we averaged the percent of Dsh at the dendritic branch points for the control RNAi (95.2%), for the Cdk5 RNAi 35855 (81.8%), and Cdk5 RNAi 104491 (81.2%). The average percent of dendritic branch points with Dsh for each experimental cross and their corresponding standard deviations (error bars) are displayed below (Figure 17). Finally, each average percent and standard deviation (control RNAi: 7.8%, Cdk5 RNAi: 16.7%, and Cdk5 RNAi 104491 14.7%) was subjected to a two-mean t-test to determine the statistical significance of the data. The p-value for the control RNAi compared to the Cdk5 RNAi 35855 value was 0.013 at the 0.05 alpha level. The p-value for the control RNAi compared to the Cdk5 RNAi 104491 value was 0.005 at the 0.05 alpha level. Finally, the calculated p-value comparing the two Cdk5 RNAi lines was 0.9 at the 0.05 alpha level. Given the statistical significance of the p values between the control and both Cdk5 RNAi line, it is possible that Cdk5 plays a significant role in localizing Dsh to the dendritic branch points of class I DA *Drosophila* neurons. Despite this, we used a relatively lenient alpha level of 0.05. With a more stringent alpha level, these results may not be deemed significant

We also performed a qualitative analysis. The images obtained, Figures 16A, 16B, and 16C, appeared to show less Dsh localizing to dendritic branch for both Cdk5 lines compared to the control. Furthermore, the change in Dsh localization between the two Cdk5 lines appeared similarly. The results of our qualitative provide some support for our quantitative conclusion that

Cdk5 regulates the localization of Dsh to dendritic branch points of class I DA *Drosophila* neurons.

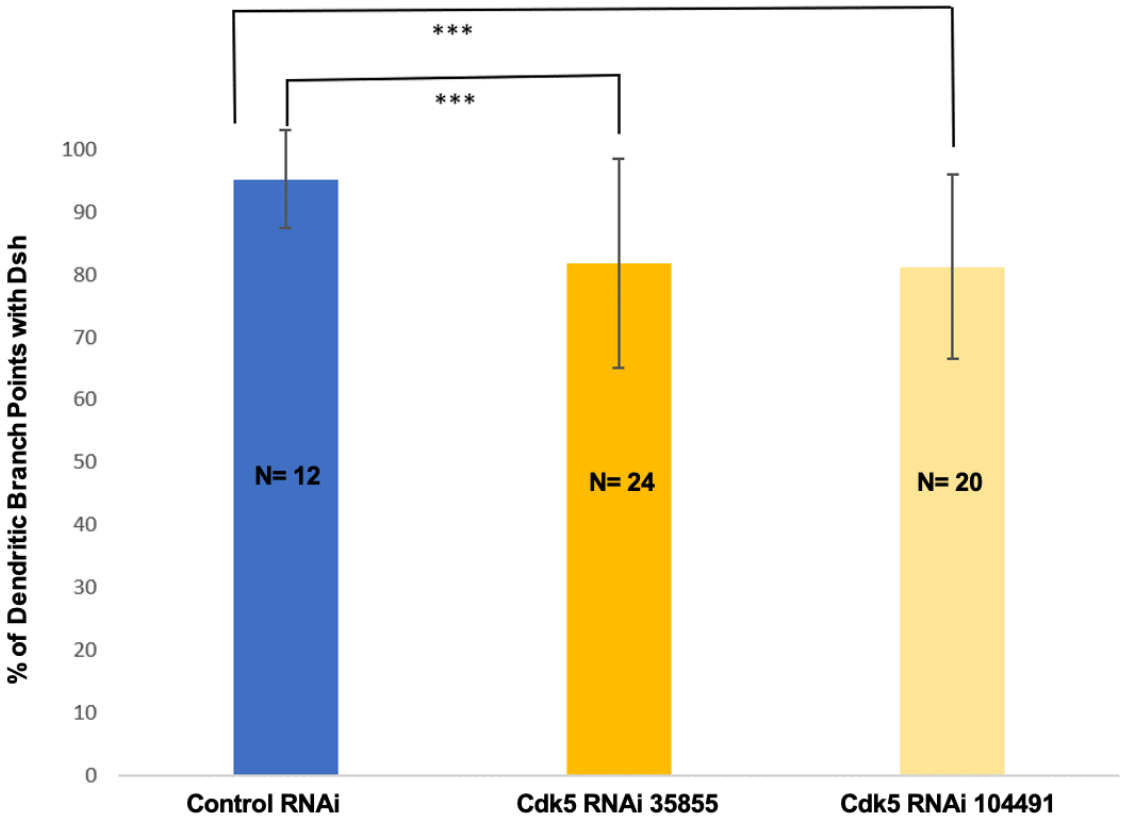

Figure 17. The average percentage and error bars of dendritic branch points localizing Dsh. The percent localized was quantified by (% of branch points with Dsh-GFP)/(Total number of branch points) X 100. A two-mean t-test was conducted to compare the control to each Cdk5 RNAi line as well as each Cdk5 RNAi line to each other. The p-values comparing the control and each Cdk5 RNAi line (35855 and 104491) was 0.021 and 0.005 at the 0.05 alpha level. The p-value comparing each Cdk5 line was 0.9 at the 0.05 alpha value.

Finally, we studied the localization of the seven transmembrane Wnt binding protein, Fz, under cellular conditions where Cdk5 is depleted. Fz was the first discovered gene of the five genes in the Fz family (Rubin G.M, et. al. 2000). More specifically, Fz is essential for distinguishing polarity in the cell (Vinson, C.R., et al. 1989). The binding of Wnt to the Fz receptor results in the recruitment and subsequent activation of the Wnt proteins, Dsh and axin, as well as other proteins

such as APC2 and GSK-3ß (Tulac S., et. al., 2003). Given the importance of Fz to transmit the Wnt signal and activate the proteins responsible for localizing MTOCs, we aimed to study the localization patterns of Fz2 (a member of the Fz G-protein coupled receptor family) using fluorescence microscopy techniques. To conduct this experiment, our lab created a *Drosophila* line containing a green fluorescently tagged Fz2 protein by following 2;3 genetic manipulation techniques. Once we created the Fz2-GFP, we crossed virgin female *Drosophila* with green fluorescence marker for the protein Fz (Fz2-GFP) with male *Drosophila* of either a control line (UAS 221, mCherry; UAS Gal4 Fz-GFP/TM6x RTNL2 RNAi 33320) or the Cdk5 RNAi line (221, mCherry; UAS Gal4 Fz-GFP/TM6 x Cdk5 RNAi 35855). Then, 3rd instar larvae were subjected to fluorescence microscopy for imaging for each cross (Figure 18A and 18B). 11 cells were imaged for the control cross, and 10 cells were imaged for the experimental Cdk5 RNAi cross.

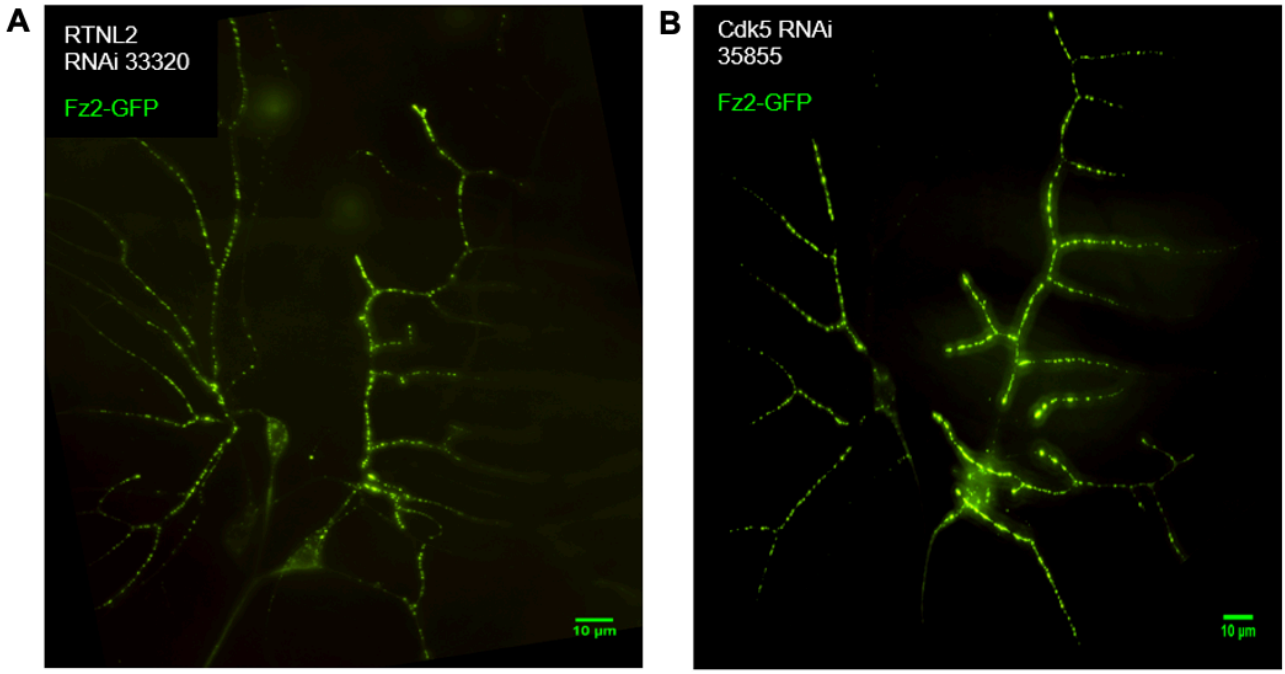

Figure 18. Fz2 localization to branch points of class I DA neurons in Drosophila following the knockdown of Cdk5 protein. (A) Example image of the control line (UAS Dicer 2, UAS

MCD8-RFP; (UAS 221 Gal 4, UAS Fz2-GFP)/TM6 x RTNL2 RNAi 33320). (B) Example image of the knockdown of Cdk5 (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Fz2-GFP)/TM6 x Cdk5 RNAi 35855).

Furthermore, quantification of the images using the computer software, FIJI (Schindelin, J. et al. 2012), allowed us to compare the control cross to the experimental Cdk5 RNAi cross. We calculated the number of "patches" (Fz2-GFP) for three segments on each sample image and then taking the average of those three numbers. Once all of the images were quantified and had a corresponding average number of "patches" per 10 microns, I took the average of the averages for the control cross and experimental Cdk5 RNAi cross. Then, we graphed the average number of "patches" per 10 microns against the control and experimental Cdk5 RNAi crosses (Figure 19). The average number of "patches" per 10 microns and corresponding standard deviation for the control cross were 9.79 and 1.56, respectively. Comparatively, the average number of "patches" per 10 microns and corresponding standard deviation for the experimental Cdk5 RNAi cross were 10.03 and 1.57, respectively. The calculated p-value following a two-mean t-test was 0.724 at the 0.05 alpha level – suggesting that depletion of Cdk5 in class I DA *Drosophila* neurons does not result in decreased localization of Fz2 to dendritic branch points.

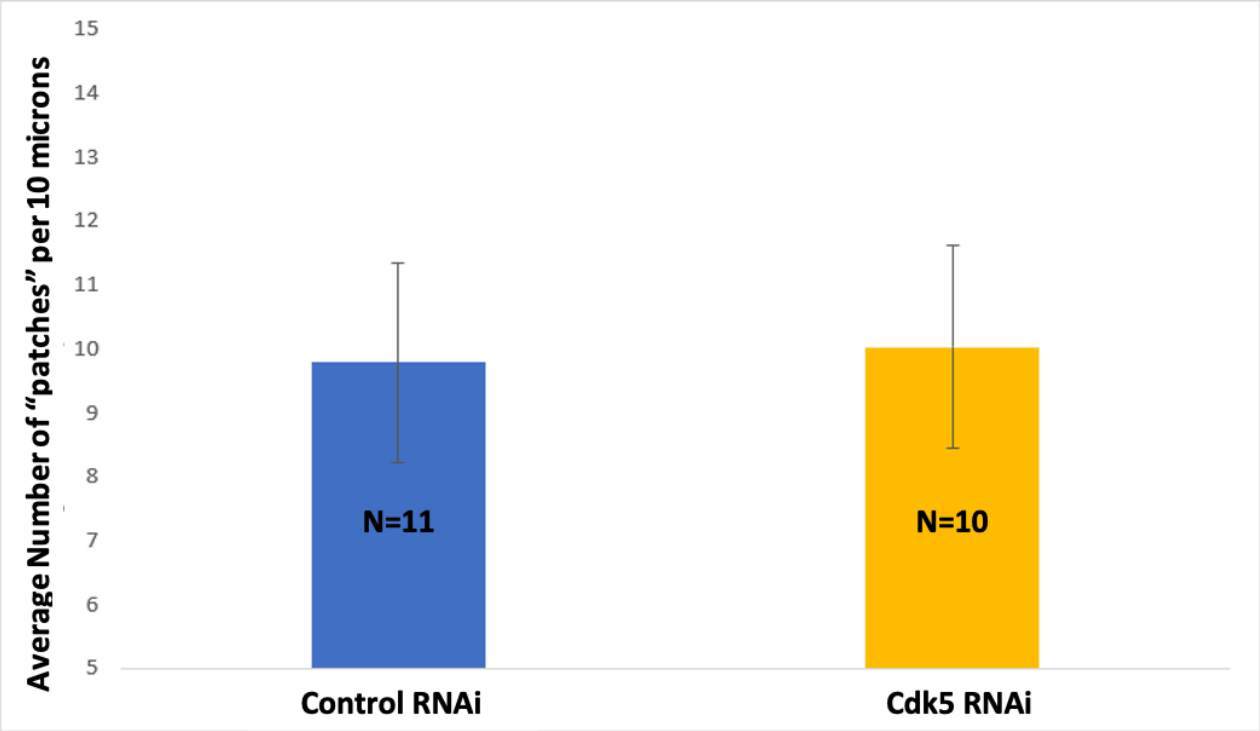

Figure 19. Average number of "patches" per 10 microns of Fz2-GFP localization to dendrites of class I DA neurons in Drosophila plotted against the control RNAi ((UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Fz2-GFP)/TM6 x RTNL2 RNAi 33320) and the experimental cross Cdk5 RNAi (UAS Dicer 2, UAS MCD8-RFP; (UAS 221 Gal 4, UAS Fz2-GFP)/TM6 x Cdk5 RNAi 35855).

Sub-Chapter 5: Localization of Cdk5 in class I DA *Drosophila* neurons

Given our goal to study the role of Cdk5 in healthy neuronal functions, we designed a localization experiment to study the positioning of Cdk5 in the cell. Due to the absence of Cdk5-GFP, we first had to create this fluorescently tagged protein using cloning techniques (Figure 20). Based on the relative size of the inserted piece of cDNA, about 850 to 900 base pairs, we expected to visualize a band at 1000 kilobases (kB) following PCR gel electrophoresis (Figure 21). By running the gel electrophoresis experiment, we could ensure that all of the cDNA was incorporated into the sequence.

Once our sequence was introduced into the *Drosophila* line, we designed a microscopy localization experiment to determine the localization pattern of Cdk5 in class I DA *Drosophila* neurons. To conduct this experiment, I collected 3rd instar larvae containing the line: UAS 221; UAS Gal4 Cdk5-GFP. 10 cells from 10 different larvae were subjected to fluorescence microscopy imaging to visualize Cdk5 localization in the cell (Figure 22). Based on the GFP visualized in the images, we concluded localization of Cdk5 to the cell body of class I DA *Drosophila* neurons.

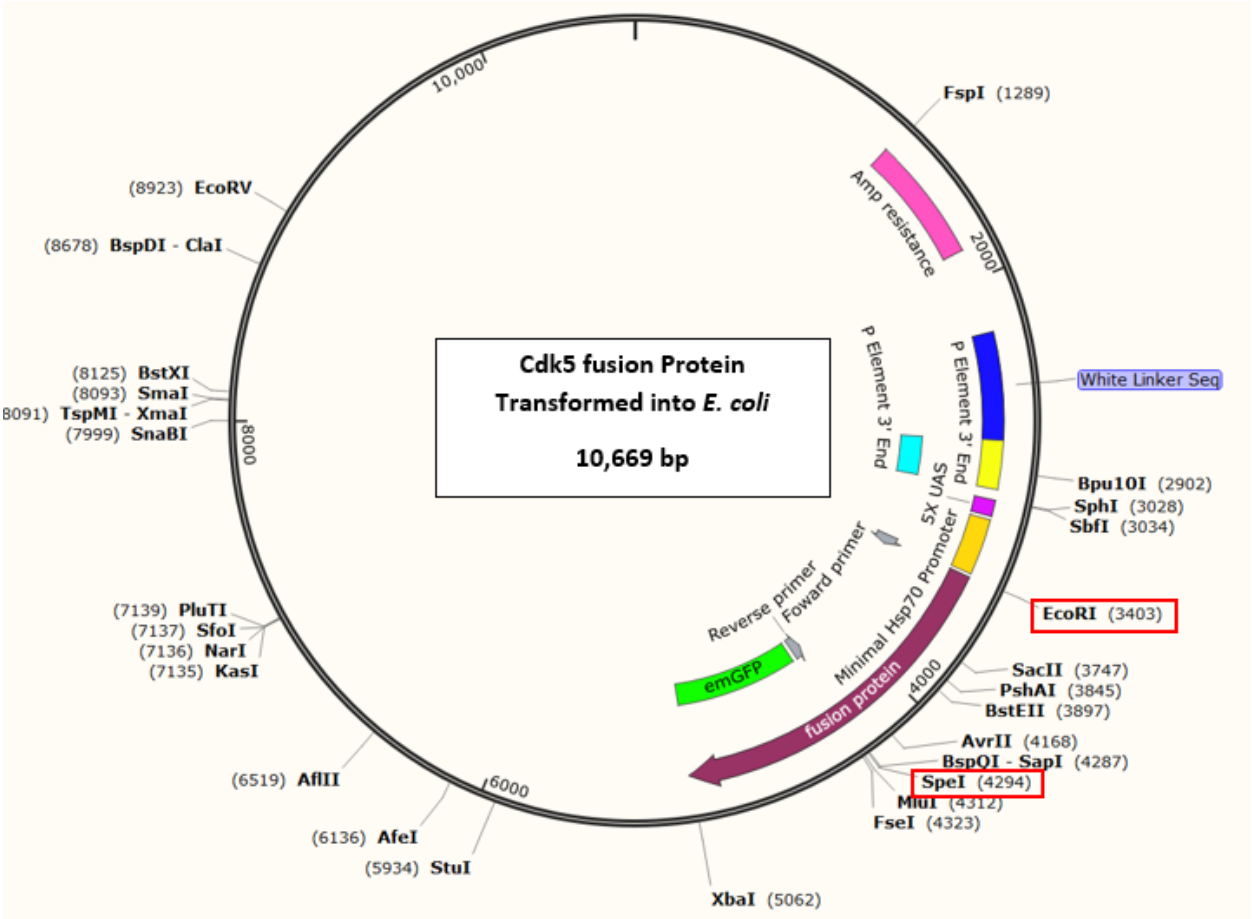

Figure 20. Vector map for plasmid ligated with Cdk5 CDS and Transfected into E. Coli. Plasmid contains Ampicillin resistance selectable marker and adds a GFP tag to the 3' end of the Cdk5 CDS. Vector was digested with EcoRI-HF and SpeI for ligation of the Cdk5 CDS. The red boxes surround the restriction enzymes chosen for the experiment.

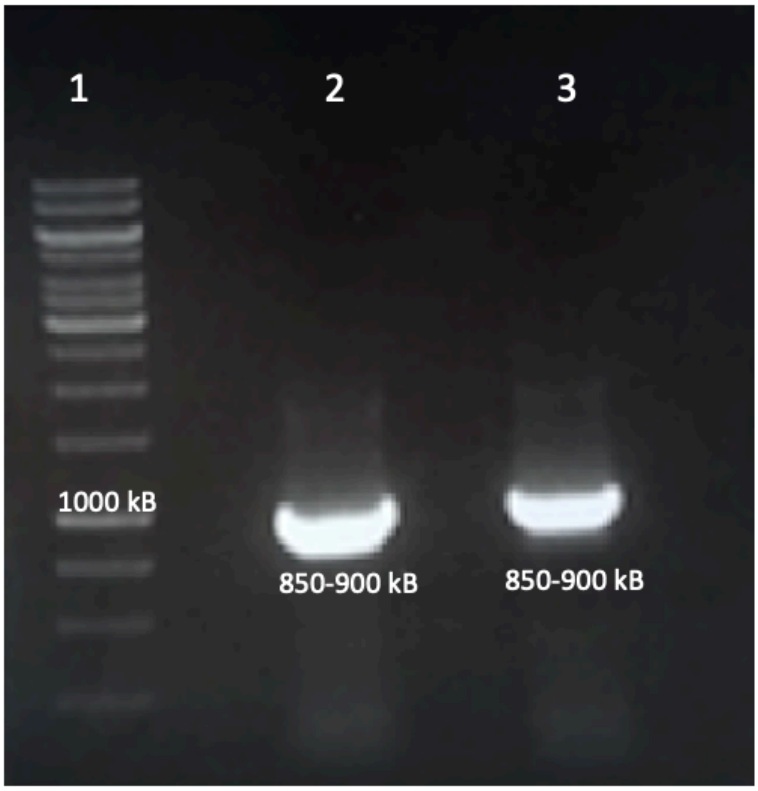

Figure 21. Lane 1: molecular ladder, Lane 2: Cdk5 cDNA sequence, and Lane 3: Cdk5 cDNA sequence. The size of the cDNA sequence was around 850-900 kB.

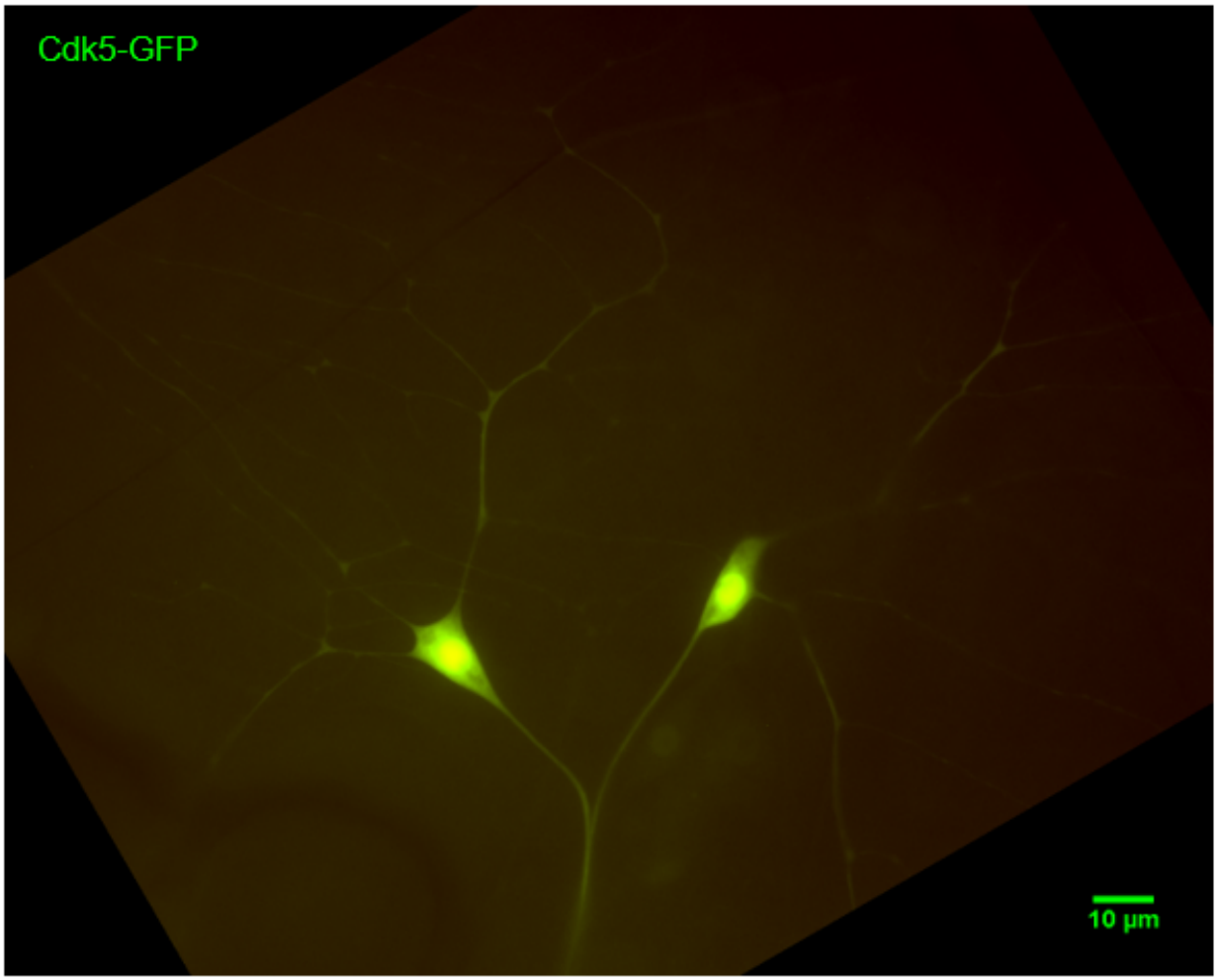

Figure 22. Localization of UAS 221; UAS Gal4 Cdk5-GFP in class I DA neurons of Drosophila.

Chapter 3

Discussion

Sub-Chapter 1: Cdk5 is required to localize γ-tubulin to dendritic branch points in DA neurons to maintain and nucleation of microtubules

Through a qualitative analysis of γ-tubulin localization, previous lab members observed a decrease in γ-tubulin localization to dendritic branch points in Cdk5 mutant organisms. Since γ-tubulin localization appeared to decrease in Cdk5 mutant cells, we developed our hypotheses that Cdk5 regulates microtubule dynamics in *Drosophila* neurons. Testing this hypothesis would allow us to better understand the role of Cdk5 in class I DA *Drosophila* neurons. Due to the role γ-tubulin plays to regulate microtubule dynamics in the cell, we hypothesized a significant change in microtubule dynamics following Cdk5 knockdown through mis-localization of γ-tubulin.

Upon studying microtubule dynamics under both control and Cdk5 depletion conditions, we discovered that minus end oriented away from the cell body microtubule polarity is disrupted upon Cdk5 depletion. This finding provided strong supporting evidence that Cdk5 regulates γ-tubulin in the cell and in turn regulates microtubule dynamics. After evaluating microtubule dynamics following Cdk5 depletion, we developed our overarching question: how does Cdk5 regulate microtubule polarity in class I DA neurons of *Drosophila*? The basis for further experimentation relied on studying the regulators of γ-tubulin localization to dendritic branch points. We wanted to study the proteins within the Wnt signaling pathway because these proteins interact to localize γ-tubulin to dendritic branch points. The findings of these two experiments provided key details to form our hypotheses: H1) Cdk5 acts on proteins upstream of Axin within

the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 5).

Sub-Chapter 2: Cdk5 knockdown does not result in decreased localization of mitochondria to dendritic branch points in class I DA *Drosophila* neurons

To address my question—how does Cdk5 regulate microtubule polarity in class I DA neurons to maintain healthy neuronal function? —it was necessary to conduct an experiment to determine the specificity of Cdk5 in the cell. Given the previous experiment, Cdk5 plays a role in localizing γ-tubulin to dendritic branch points and maintaining polarity of microtubules in the dendrites, we aimed to study the specificity of Cdk5 to accomplish this in the dendrites. Assessing the mitochondrial localization when Cdk5 levels were depleted allowed us to determine its specificity for microtubule regulation. If levels of mitochondria were significantly different in Cdk5 mutant cells, we could assume a general role of Cdk5 in the cell because mitochondrial are not responsible for microtubule regulation. This could indicate Cdk5 functions to activate dynein, a minus end directed microtubule motor protein, to aid directing proteins to dendritic branch points. If the levels of mitochondria were unchanged in the Cdk5 mutant cells, we could assume Cdk5 functions specifically on proteins responsible for regulating microtubules in dendrites.

From our localization assays of mitochondria, we determined insignificant findings because mitochondrial localization appeared unchanged between Cdk5 mutant cells and the control cells. As a result, we concluded that Cdk5 likely plays a specific role in localizing γ-tubulin to dendritic branch points and maintaining polarity of microtubules in the dendrites. Since the results of our mitochondrial localization assay revealed insignificant results, we could

support our hypotheses with Cdk5 functioning specifically on microtubule regulators: H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 5). From this experiment, we began studying more specific regulators of γ-tubulin localization.

Sub-Chapter 3: Cdk5 knockdown does not result in decreased localization of APC2 to dendritic branch points in class I DA *Drosophila* neurons

To further elucidate the role Cdk5 plays in maintaining healthy neuronal function in class I DA neurons, we assessed the localization of APC2 to dendritic branch points in Cdk5 null cells. This experiment provided a way to address a possible pathway Cdk5 functions in to localize γ-tubulin to the branch points and regulate microtubule polarity. If the results of our APC2 localization assay appeared statistically significant in the Cdk5 knockdown cells, this could indicate Cdk5 functions through an alternative pathway—outside of the Wnt signaling pathway—to regulate microtubule polarity. In contrast, if there is no significant difference in APC2 localization, Cdk5 possibly functions within the Wnt signaling pathway—supporting our hypotheses (Figure 5).

From our fluorescence microscopy assay, we determined insignificant results for APC2 localization. Therefore, Cdk5 knockdown does not result in decreased APC2 localization to dendritic branch points. One possibility could be that Cdk5 specifically functions to regulate Wnt signaling proteins. Another possibility could be that Cdk5 has a role in regulating Wnt proteins in addition to other proteins, but not APC2. The latter of these possibilities led us to move into further experimentations analyzing protein localization assays of proteins constituting the Wnt signaling pathway.

Sub-Chapter 4: Cdk5 is required to localize Axin and Dsh to dendritic branch points but not required to localize Fz to dendritic branch points in class I DA *Drosophila* neurons

Following the results from our previous experiments, we began studying the localization patterns of proteins within the Wnt signaling pathway (Axin, Dsh, and Fz) from cells expressing lower levels of Cdk5. We started with the first upstream protein from γ-tubulin, Axin, for our localization experiments. Assessment of Axin localization directly addressed our hypotheses-- H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 5). If the levels of Axin varied in Cdk5 knockdown cells, this would support our hypotheses. If the levels of Axin remained stable comparatively between Cdk5 knockdown and control cells, this would refute both hypotheses.

From our localization assays of Axin, we concluded a significant role of Cdk5 for localizing Axin to dendritic branch points. Since Axin regulates γ-tubulin localization, this finding makes sense since we concluded Cdk5 has a role in γ-tubulin localization and regulating microtubules at dendritic branches. As a result, our hypotheses-- H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway or H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway (Figure 5)—remained supported and we could assess localization of patterns of upstream proteins—relative to Axin—to further determine how Cdk5 specifically regulates Axin localization.

Next, we sought out to discover the localization pattern of Dsh to dendritic branch points when Cdk5 levels were depleted in the cell. As the first upstream protein relative to Axin in the Wnt signaling pathway, Dsh localization could help us to support one of our alternate hypotheses while refuting the other. If Dsh localization remained relatively similar between the Cdk5

knockdown cells and the control cells, this would support H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway insignificantly and refute H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway. In contrast, if Dsh localization varied significantly between Cdk5 knockdown and control cells, this would support H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway and refute H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway insignificantly.

Our experiment studying Dsh localization to dendritic branch points following depletion of Cdk5 showed a significant* decrease in Dsh localization. Dsh lies upstream of Axin within the Wnt signaling pathway to regulate γ-tubulin localization to dendritic branch points. Therefore, the significant decreased localization of Dsh to dendritic branches in Cdk5 depleted cells supports H1) Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway and refute H2) Cdk5 acts directly on Axin through a parallel pathway outside of the Wnt pathway insignificantly. Given that the statistical test ran at the 0.05 alpha level, the possibility of no statistically significant difference of Dsh localization following Cdk5 knockdown still remains and additional experiments studying Dsh localization at a lower alpha level could provide more convincing results.

Moving forward, we designed an experiment to analyze Fz localization to dendrites with cells expressing lower levels of Cdk5. Using the Fz-2-GFP line created in our lab, we captured images of Fz localization in the dendrites (Figure 18A and 18B). In doing so, we found that Cdk5 does not regulate the localization of Fz in dendrites. Fz acts as the transmembrane receptor responsible for binding the Wnt signal and transmitting the signal through the cell by recruiting Wnt proteins (Dsh, APC2, Axin, GSK3ß) Given this, and the results of our APC2 localization

assay, we expected Fz localization to be unaffected by the knockdown of Cdk5. This supports the hypothesis stating Cdk5 acts on proteins upstream of Axin within the Wnt signaling pathway.

Sub-Chapter 5: Cdk5 localizes to the cell body of class I DA *Drosophila* neurons

The result of our previous experiments prompted us to discover the localization of Cdk5 in the cell. Determining the localization of Cdk5 in the cell opens the door to understanding the role it plays in maintaining healthy neuronal functions. Furthermore, knowing where Cdk5 localizes provides a basis for developing novel experiments to determine the significance it has in neurons. We utilized C-terminal infusion cloning experiment from the cDNA of the Cdk5 gene to create a green fluorescently tagged Cdk5 protein (Cdk5-GFP). Using fluorescence microscopy techniques, we discovered that Cdk5 localizes to the cell body of class I DA neurons of *Drosophila*. Given that Cdk5 clearly plays a role in regulating the localization of Wnt signaling proteins in the dendrites of class I DA cells, its localization to the cell body could indicate it plays other non-specific roles in these neurons. Future experiments should work to elucidate the roles Cdk5 plays in the cell in addition to its regulation of the Wnt signaling pathway. Additionally, moving forward, specific experiments to determine a direct activation of the Wnt proteins—Dsh and Axin—or whether activation of an intermediate protein leads to the Wnt protein activation could reveal the specificity of Cdk5 in regulating healthy neuronal functions.

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ACADEMIC VITA

Katelyn Rudisill

RESEARCH EXPERIENCE

The Rolls Lab, Penn State University — ***Undergraduate Researcher*** July 2017 – PRESENT
-Study a specific protein kinase to understand its role in healthy neuronal functions using *Drosophila.*
-Learn laboratory techniques, such as manipulating *Drosophila* genetics, fluorescence microscopy imaging and analysis, and molecular cloning.
-Refine critical thinking skills, problem-solving skills, and scientific writing skills through designing experiments, forming hypotheses, and writing a thesis paper.
-Aim to understand proteins responsible for healthy neuronal function and their role in neurodegenerative diseases.

Perdana University Royal College of Surgeons in Ireland — *Co-Investigator* May 2020 – PRESENT
-Contribute to the scientific community by examining medical students' mental health and online learning experiences during the COVID-19 pandemic on an international, multi-center scale (United States, Europe, Middle East, and Asia).
-Enhance scientific communication and proposal writing skills
-Learn how to apply for IRB approval and create a modified survey questionnaire.
-Seek to improve medical education for future medical students and minimize mental health problems associated with online medical education learning.

CLINICAL EXPERIENCE

C2 Solutions Inc./Scribe America/Internists of Central PA - *Medical Scribe* May 2018 – November 2019
-Collaborated with physicians and other medical scribes to complete electronic medical records.
-Broadened my medical knowledge through new medical terminology and patient cases.
-Gained an understanding of patient-physician relationships and critical thinking skills required as a physician.
-Taught new medical scribes how to complete a patient's electronic medical chart.

PHILANTHROPIC/VOLUNTEER EXPERIENCE

Student Pad Project, Penn State University – ***Co-Founder & Vice President*** May 2019 – *PRESENT*
-Collaborate with 30 club members to set up fundraisers to raise about $10,000 to establish self-sufficient sanitary pad making sites for women in rural villages of developing countries.
-Teach receiving women to sell any extra pads they produce to women in surrounding villages while training them in proper menstrual hygiene practices.
-Enhance leadership skills by delegating tasks to club members and leading club meetings.

Big Brothers Big Sisters, Center County Youth Resources Bureau **- *Big Sister***
-Mentor for young girls in the State College Area for two hours each week.
-Counsel my "little" sister to treat others appropriately and communicate their frustration. • Offer support and a listening ear.

The Arthritis Foundation, Camp JRA at Camp Victory – ***Camp Counselor***
-Educated children on how to properly communicate their disease.
-Assisted with organizing camp activities; fostered cooperation among campers and staff

Chemistry Department, Penn State University— ***Learning Assistant*** January 2020 – Spring 2021
-Tutored 5-20 students in organic chemistry topics by attending class and hosting individual office hours once a week for 2 hours; served as a liaison between professor and students.

LEADERSHIP EXPERIENCE

Presidential Leadership Academy, Penn State **– Member** May 2018 – PRESENT
-Gain cultural awareness through in-class discussions and embedded field trips.
-Expand my leadership and communication skills through class projects, presentations, and events.

ENTREPRENURIAL EXPERIENCE

Paradise MCAT Prep – Co-Founder & CEO June 2020 – PRESENT
-Address the need for an all-encompassing, innovative, and affordable MCAT preparation plan for students.
-Designed and marketed a webpage and social media accounts, leading to close to $2,000 in sales to date.
-Created innovative test preparation materials.

EDUCATION

Pennsylvania State University - Schreyer Honors College, State College, PA — ***B.S. Biochemistry & Molecular Biology*** July 2017 – May 2021
-Academic Achievements & Awards: Dean's list 2017-2021, Evan Pugh Junior & Senior Scholar Award, Erikson Discovery Grant, Academic Scholarships totaling $13,100: Arthur Anderson, Beirig Renaissance, Class of 1922 Memorial, Presidential Leadership Academy, Ready to Succeed, Schreyer Family Honors, and the Schreyer International Study.

Embedded Japanese Culture Study Abroad Trip, Penn State University May 2019 – June 2019
-Experienced life in Tokyo, Japan, with ten other Penn State Students for three weeks.
-Broadened cultural awareness by learning Japanese culture and traditions.

Erickson Discovery Grant
-Received $3,500 for my independent research project studying a specific protein kinase and its role in maintaining healthy neuronal function in the model organism *Drosophila.*