Cyclin-dependent kinase-5 regulates the polarized trafficking of dense-core vesicles in *C. elegans* motor neurons

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Abstract

Cyclin-dependent kinase-5 (CDK-5) is a serine/threonine kinase with many important functions in neurons, including regulation of development, protein transport, synaptic transmission and plasticity, and neurodegeneration. Aberrant activation of CDK-5 can cause neuronal death, and inhibition of CDK-5 is protective in animal models of Alzheimer's disease, prion protein disease, and Huntington's disease. In this thesis, I use *C. elegans* to investigate the role of CDK-5 in regulation of neuropeptide trafficking and synaptic transmission. In Chapter 2, I identify a novel role for CDK-5 in the regulation of polarized trafficking of dense-core vesicles. Dense-core vesicles (DCVs) release neuropeptides, which are key regulators of synaptic strength and plasticity, but little is known about the regulation of DCV transport. I utilize a fluorescently-tagged neuropeptide (INS-22::Venus) to visualize DCVs in DA and DB cholinergic neurons, which have a well-defined axon and dendrite. In wild-type animals INS-22::Venus localizes primarily to axons, but *cdk-5* mutants have reduced axonal DCVs and increased dendritic DCVs, indicating that CDK-5 promotes the polarized distribution of DCVs to the axon. I find that CDK-5 is not required to establish axon/dendrite polarity, but instead acts at the cell body to inhibit the trafficking of DCVs into the dendrite. Mutations in cytoplasmic dynein (*dhc-1*) completely block the increase in dendritic DCVs observed in *cdk-5* mutants, suggesting that CDK-5 inhibits dynein-mediated trafficking of DCVs into dendrites. In Chapter 3, I identify cell-type specific differences between DA and DB motor neurons. In DA neurons, *cdk-5* mutants have increased dendritic DCV markers without a corresponding change in axonal DCVs, suggesting CDK-5 has

independent functions in the axon and dendrite. DB motor neurons, in contrast, have decreased axonal DCVs in *cdk-5* mutants. Similarly, time-lapse imaging shows that DCV trafficking to the axon is altered in DB neurons, but not DA neurons, in *cdk-5* mutants. Finally, I investigate the role of CDK-5 in synaptic transmission at the neuromuscular junction (NMJ) using a paralysis assay based on an inhibitor of acetylcholinesterase, aldicarb. These studies reveal that CDK-5 promotes acetylcholine release at the NMJ and lay the groundwork for future studies to identify novel CDK-5 substrates and regulators that promote synaptic transmission.

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CHAPTER 1

Introduction

Polarized transport of neuronal cargoes

In order for neurons to convey information in a single direction, they must be polarized into two distinct subcellular compartments: one specialized for signal reception (the somatodendritic compartment) and one specialized for signal transduction (the axon). Each compartment has a unique structure and repertoire of proteins specific to its function. Dendrites are highly branched to receive multiple synaptic inputs from upstream neurons. They contain neurotransmitter receptors and all the molecular machinery necessary for their regulation, including scaffolding proteins, associated ion channels, endosomes, and mRNA complexes. Axons, on the other hand, usually have a single narrow process that can extend long distances, that ends in branches called collaterals which contact downstream targets. Axons contain voltage-gated ion channels required to propagate action potentials and all the proteins essential for activity-dependent synaptic vesicle release. In order to maintain this asymmetric distribution of proteins, neurons must be able to distinguish axonal cargoes from dendritic cargoes and polarize their trafficking. This raises the intriguing question of how neuronal cargoes discriminate the axon from the dendrite.

Theoretically, polarization of proteins to a single neuronal compartment could be achieved through multiple mechanisms. The simplest strategy for polarization is direct trafficking of each cargo to its appropriate location. Another potential strategy is to non-specifically transport proteins to all parts of the cell, and afterwards selectively remove them from the inappropriate compartment. Neurons, it seems, employ both strategies (Sampo et al., 2003). Some dendritic cargoes, including the transferrin receptor and the metabotropic glutamate receptor mGluR1a, are directly trafficked to dendrites (Burack et al., 2000; Horton and Ehlers, 2003). Likewise, some axonal cargoes, including the voltage-gated potassium channel Kv1.2 (Lai and Jan, 2006) and the neuron-glia cell adhesion molecule (NgCAM)(Sampo et al., 2003), traffic directly to axons. However, some axonal cargoes,

including the voltage-gated sodium channel $Na_V1.2$ (Lai and Jan, 2006) and the synaptic vesicle- associated protein Synaptobrevin/VAMP2 (Sampo et al., 2003), are initially trafficked globally to axons and dendrites, then subsequently endocytosed only from the dendritic compartment, resulting in overall polarization to the axon. These findings imply that the two different subcellular compartments have their own, separate mechanisms for removal of incorrect proteins, and their own unique cues that can be recognized by the cell's transport machinery.

The role of the cytoskeleton in neuronal polarity

The organization of the cytoskeleton can act as a cue that guides axonal or dendritic cargoes to their destination. Microtubules themselves are polarized into a quickly polymerizing "plus" end and a more slowly polymerizing "minus" end. Microtubule orientation differs between the axon and the somatodendritic compartment (Baas et al., 1988) and could serve as a means for cargoes to discriminate axon from dendrite. In the axon, the vast majority of microtubules are oriented with their plus-ends distal to the cell body (Baas et al., 1988; Stone et al., 2008). In the dendrites of mammalian neurons, microtubules are of mixed polarity in the proximal dendrite and plus-end out in the distal dendrite (Baas et al., 1988). *Drosophila* dendrites, in contrast, have most microtubules oriented minus-end out (Stone et al., 2008). The exact orientation of microtubules in the axon and dendrites has yet to be determined in *C. elegans*, but a recent study suggests that the pattern of all plus-end out microtubules in the axon is conserved in *C. elegans* (Ou et al., 2010). This pattern of microtubule orientation, combined with motor proteins that track specifically towards plus-ends or minus-ends, can theoretically provide a basis for polarized transport. Because minus-end out microtubules are only present in dendrites, cargoes carried by minus-end directed motors should be able to enter dendrites but would be

excluded from axons. Similarly, plus-end out motors could enter both axons and dendrites, but would move bi-directionally in dendrites and uni-directionally in axons, eventually resulting in an overall polarization towards the axon.

However, cargoes appear to be able to detect differences in axonal and dendritic microtubules that go beyond the differences in microtubule orientation. Post-translational modifications of tubulin differ between the two subcellular compartments and can modify motor protein binding and mobility. α-tubulin can have its C-terminal tyrosine cleaved off, be acetylated at lysine 40, and have polyglutamate chains added to glutamate 445 (Fukushima et al., 2009). De-tyrosinated tubulin is enriched at the ends of microtubules in the axon initial segment, but is not found on dendritic tubulin (Konishi and Setou, 2009). The motor head of the plus-end directed motor Kif5 (kinesin-1) can preferentially bind to this axonal de-tyrosinated tubulin (Konishi and Setou, 2009). A mutation in Kif5 that causes the motor to bind non-specifically to all tubulins (regardless of tyrosination level) disrupts axon-specific transport of Kif5 cargoes, suggesting Kif5 recognizes axonal microtubules through a de-tyrosinated tubulin-binding mechanism (Konishi and Setou, 2009). Similarly, acetylation is required for binding of Kif5 to tubulin and can enhance Kif5-mediated transport (Fukushima et al., 2009). Low levels of acetylated tubulin can result in reduced transport of brain-derived neurotrophic factor (BDNF)-containing vesicles, which may lead to neuronal death and be a key event in the pathogenesis of Huntington's Disease (HD) (Dompierre et al., 2007). Polyglutamylation is necessary for the binding of the microtubule-associated protein MAP2, which is required for activity-dependent changes in glycine receptor trafficking (Maas et al., 2009). Increased neuronal activity increases polyglutamylation of tubulin, which in turn increases MAP2 binding, leading to a decrease in Kif5-mediated delivery of glycine receptors to postsynaptic sites (Maas et al., 2009).

This finding by Maas et al (2009) that altering MAP2 binding affects the targeting of postsynaptic cargoes to synapses highlights the role that microtubule-associated proteins (MAPs) play in regulation of transport. Binding of MAPs to microtubule filaments can facilitate polymerization and bundling of microtubules and can determine the distance between adjacent microtubules (Alberts, 2002). Additionally, MAPs in both the axon and dendrite can inhibit motor motility, potentially by competing with motors for binding sites to microtubule filaments (Dehmelt and Halpain, 2005). Tau is a MAP that binds specifically to axonal tubulin. When a kinesin motor moving along a microtubule encounters a cluster of tau protein, the kinesin is more likely to pause (Dixit et al., 2008). When a dynein motor encounters tau, the motor is more likely to reverse directions (Dixit et al., 2008). Asymmetric binding of MAPs in the axon versus the dendrite could therefore act as an inhibitory or permissive cue important for polarized transport.

In addition to the microtubule cytoskeleton, actin networks may also be essential for the polarized trafficking of dendritic proteins. Recent studies indicate that an intact actin network is required for polarized trafficking (Lewis et al., 2009; Song et al., 2009) and that high molecular weight compounds are prevented from entering axons, suggesting the existence of an actin "filter" that can prevent entry into the axon. Moreover, binding to the actin-based motor myosinVa was found to be both necessary and sufficient to target transmembrane proteins to the dendrite (Lewis et al., 2009). These findings led to the hypothesis that the axon initial segment contains a filter of actin filaments pointing away from the axon. If a myosin bound to a dendritic cargo encounters the filter, it will carry its cargo along the actin filaments and away from the axon. In addition to this internal actin filter, there also appears to be a filter within the plasma membrane at the axon initial segment (AIS) that prevents membrane proteins from diffusing into and of out the axon (Winckler et al., 1999). When fluorescently tagged phospholipids are inserted into the

axonal membrane, they can diffuse up to, but not beyond, the AIS, demonstrating the existence of a diffusion barrier (Rasband, 2010). This implies that in addition to the microtubule highways that aide the movement of cargoes into the axon, there are also cytoskeletal and membrane-bound structures that function as road blocks to prevent dendritic cargoes from inappropriately entering the axon.

Molecular motors that mediate polarized trafficking

Collectively, the asymmetry of microtubule orientation, posttranslational tubulin modifications, MAPs, and the actin network provide a framework for motor proteins to carry cargoes in a polarized way. Motors belonging to the kinesin and dynein families mediate microtubule-based transport and are responsible for carrying neuronal cargoes to pre- and postsynaptic sites. Kinesins may be plus- or minus-end directed, depending on the location of their motor domain. Kinesins with N-terminal motor domains, including Kif5 (kinesin-1)/UNC-116 and Kif1A(kinesin-3)/UNC-104, are plus-end directed, while kinesins with C terminal motor domains, such as KifC2/KLP-17, are minus-end directed (Hirokawa and Takemura, 2004). Cytoplasmic dynein is a hexomeric AAA-ATPase that can move bi-directionally on microtubules *in vitro*, but acts primarily as a minus end-directed motor *in vivo* (Ross et al., 2006).

Based on the orientation of microtubules in axons and dendrites, a simplified model of polarized transport might predict that all axonal cargoes are transported by plus-end directed motors while all dendritic cargoes are carried by minus-end directed motors. This model would also predict that anterograde traffic (cargoes moving away from the cell body) in the axon is mediated by plus-end directed motors, while retrograde transport (cargoes moving back towards the cell body) is carried out by minus-end directed motors. Many motors indeed have functions consistent with this model. The minus-end directed kinesin

KifC2 carries multi-vesicular bodies into dendrites (Hirokawa and Takemura, 2005). Minus-end directed dynein is required for the transport of glycine receptors and AMPA-type glutamate receptor subunit GluR2 to dendrites (Hirokawa et al., 2010; Kapitein et al., 2010a). Plus end-directed Kif1A mediates the axonal transport of synaptic vesicles and dense core vesicles (DCVs) in mammals, flies, and *C. elegans* (Barkus et al., 2008; Hall and Hedgecock, 1991; Jacob and Kaplan, 2003; Lo et al., 2011; Okada et al., 1995; Pack-Chung et al., 2007; Zahn et al., 2004). Kif1Bβ, another plus-end directed motor, also transports synaptic vesicles to axons in mammals (Hirokawa et al., 2010). Kif5 and Kif1Bα are also required for the transport of mitochondria down axons (Hirokawa and Takemura, 2005). Kif5 additionally transports syntaxin-containing vesicles, amyloid precursor protein, and apolipoprotien receptor 2 to axons and has been implicated in synaptic vesicle transport in *C. elegans* (Byrd et al., 2001; Hirokawa and Takemura, 2005). Dynein appears to be the major retrograde motor for axonal cargoes in mammals, *Drosophila*, and *C. elegans* (Koushika et al., 2004; Levy and Holzbaur, 2006). Together, these findings provide multiple examples consistent with the idea that plus-end motors are adapted for anterograde axonal trafficking and minus-end motors are adapted for anterograde dendritic trafficking and retrograde axonal trafficking.

However, inconsistent with the prediction that the direction of motor movement determines whether it will carry axonal or dendritic cargoes, there are plus end-directed motors that transport dendritic cargoes. Kif17 is a plus-end directed kinesin that transports NMDA receptors to dendrites (Setou et al., 2000). Interestingly, in addition to its role as an axonal motor, Kif5 can also act as a dendritic motor. It is required for transport of the AMPA receptor subunit GluR2 (Setou et al., 2000) and mRNA-containing granules into dendrites (Kanai et al., 2004). This demonstrates that while the microtubule orientation in the axon

prevents minus-end motors from mediating anterograde axonal transport, plus-end directed motors can carry proteins to either axon or dendrite.

The dual role of the plus-end directed kinesins in axonal and dendritic transport raises two interesting questions: do motor heads distinguish dendritic microtubules from axonal microtubules or can cargoes affect their motor's ultimate destination? By creating motor mutants without auto-inhibitory tail domains and cargo binding domains, researchers can determine where motors would travel in the absence of cargo. Similarly, modified motors that bind microtubules but are not processive can be used to find the microtubule sites to which motors preferentially bind. Results from such experiments indicate that some, but not all, kinesins can bind microtubules preferentially in the axon initial segment (Nakata and Hirokawa, 2003; Ou et al., 2010). UNC-104, a carrier of axonal cargoes, accumulates specifically in axons in *C. elegans* when its tail is removed (Ou et al., 2010). On the other hand, tail-less Kif17, which carries dendritic cargoes, accumulates in both the axon and dendrites and motor-dead Kif17 binds non-specifically to axonal and dendritic microtubules (Nakata and Hirokawa, 2003). Interestingly, tail-less Kif5, which transports axonal and dendritic cargoes, accumulates only in axons and motor-dead Kif5 binds specifically to the axon initial segment (Nakata and Hirokawa, 2003). Thus, compartment-specific binding of kinesins can assist in polarized transport in the case of UNC-104, is not necessary for polarized transport in the case of Kif17, and must be overcome during dendritic transport in the case of Kif5.

These findings show that axon- or dendrite-specific binding of motors to microtubules can assist polarized transport, but is not the only determinate of transport directionality, suggesting that perhaps the interaction between motors and their cargoes affects the motor's ultimate destination. In support of this idea, Setou et al (2000) demonstrated that the localization of a motor can be specified by the cargo to which it is bound. The normally

axonal Kif5 can be re-directed to the somatodendritic compartment by overexpression of the GluR2-binding protein GRIP-1, implying that cargos can steer their motors to the correct subcellular compartment.

The finding that GRIP-1 binding steers Kif5 into dendrites demonstrates that polarized transport can be regulated by the adaptor proteins or scaffolding proteins that link motors to cargoes. GRIP-1 is the motor adaptor for Kif5 in the dendrite, but JNK interacting proteins (JIPs) act as the Kif5 adaptor for axonal cargoes (Arimoto et al., 2011; Byrd et al., 2001; Sakamoto et al., 2005). JIPs bind APP and APOE2 to Kif5 in mammals and act as an adaptor for synaptic vesicle binding to UNC-116/Kif5 in *C. elegans* (Arimoto et al., 2011; Byrd et al., 2001; Sakamoto et al., 2005). UNC-104 binds synaptic vesicles through an adaptor called DENN/MADD, which links UNC-104 to the synaptic vesicle through associations with the small GTPase Rab-3 (Niwa et al., 2008). Direct binding of UNC-104's plextrin homology (PH) domain to liposomes, however, has also been proposed to link the motor to its cargoes (Klopfenstein and Vale, 2004). Motor binding to adaptor proteins may facilitate motor processivity as well as provide a link between motor and cargo (Miller et al., 2005; Schroer, 2004; Wagner et al., 2009). SYD-2/Liprin-α binds to UNC-104/Kif1A in mammalian neurons and *C. elegans* and can bind to Kif5/kinesin-1 in *Drosophila* (Miller et al., 2005; Shin et al., 2003; Wagner et al., 2009). This interaction regulates the relative amount of anterograde and retrograde movements of UNC-104 and Kif5's cargoes, and increases UNC-104 velocity and Kif5 run-length (Miller et al., 2005; Wagner et al., 2009). Dynein associates with a large multi-subunit protein complex called dynactin. Binding of dynein to dynactin is necessary both to link dynein to its cargoes but also to enhance dynein processivity (Schroer, 2004).

Adaptor proteins can bind more than one type of motor, a process that may be important for activating motors and allowing bi-directional motility (Ally et al., 2009; Muller et

al., 2008). Dynactin has been demonstrated to not only bind and regulate dynein, but can also bind to kinesin-2 (Berezuk and Schroer, 2007). The adaptor protein Huntingtin (htt) can bind Kif5, Kif1A, and dynein, and potentially bring more than one motor into a protein complex (Caviston and Holzbaur, 2009). This allows attachment of an anterograde and a retrograde motor to the same cargo, which can facilitate the bi-directional movement of that cargo.

In summary, scaffolding proteins can attach cargoes to motors that preferentially traffic to axons or dendrites in order to polarize protein transport, or the scaffolding protein/cargo itself can steer the motor to the correct compartment. Removal of proteins from the incorrect subcellular compartment also results in polarized protein localization. Both of these are cell-intrinsic mechanisms, but neuronal polarity can also be influenced by extra-cellular signaling. In *C. elegans*, the netrin UNC-5 is secreted from the ventral side of the body and acts an inhibitory cue that prevents the trafficking of axonal cargoes to dendritic processes in the ventral nerve cord (Poon et al., 2008). This suggests that signaling cascades responding to extra-cellular cues may act locally in one part of the neuron to regulate polarized trafficking.

While a great deal of research has been performed to uncover the mechanisms that control polarized trafficking in neurons, the primary focus has been on membrane proteins, including neurotransmitter receptors, ion channels, and synaptic vesicle associated proteins. In contrast, we know relatively little about how the neuron polarizes the trafficking of soluble cargoes destined for secretion through the regulated secretory pathway. Vesicles containing lumenal cargoes can undergo subcellular compartment-specific secretion (Fisher et al., 1988; Landry et al., 2003). This requires two different mechanisms to polarize secretory vesicle trafficking, one for axonal cargoes and one for dendritic, and also a means of discriminating which mechanism should be employed. This is a potentially challenging

task, considering that the proteins destined for secretion are separated from the motors that transport them by the vesicle membrane. In addition, unlike transmembrane proteins, secreted cargoes cannot undergo non-specific trafficking to both axons and dendrites, and then subsequently be endocytosed from the inappropriate compartment. Additionally, secreted cargoes diffuse away after release and need to be replenished by continual trafficking of new proteins. Therefore, the regulation of polarized trafficking of secreted proteins poses an interesting research question that has yet to be addressed.

Dense-core vesicle and neuropeptide function

One secreted cargo essential for nervous system function are the neuropeptides that are released from dense-core vesicles (DCVs). In addition to release of classical neurotransmitters, it has become clear that most neurons also release at least one type of neuropeptide (Mains and Eipper, 1999). Neuropeptide release regulates synaptic transmission, neuronal activity, and behavior in animals ranging from *C. elegans* to mammals. In fact, despite the drastic difference between the size and complexity of the nematode and the mammalian nervous system, the number of predicted neuropeptides in the *C. elegans* repertoire appears to rival the number of neuropeptides identified in mammals (Li, 2005). There are three neuropeptide gene families in *C. elegans*: the FMRF-amide related peptides (*flp* genes), insulin-like neuropeptides (*ins* genes), and neuropeptide-like proteins (*nlp* genes). (Li et al., 1999; Nathoo et al., 2001; Pierce et al., 2001). There are currently 31 *flp* genes, 40 *ins* genese, and 33 *nlp* genes identified in the genome, summing up to a total of 104 predicted neuropeptide genes encoding roughly 250 distinct peptides (Li, 2006).

Neuropeptides undergo calcium-dependent release in response to neuronal activity and, once released, can bind receptors on postsynaptic neurons (synaptic signaling),

adjacent neurons (paracrine signaling), or more distant cellular targets (endocrine signaling) (Mains and Eipper, 1999). Additionally, neuropeptides can bind to receptors on the neuron from which they were released (autocrine signaling). Some neuropeptides fit the criteria for a classical neurotransmitter, while others modulate the activity of other neurotransmitters (Mains and Eipper, 1999). Modulation of neurotransmitter function and neuronal activity by neuropeptides can shape neuronal network firing patterns and ultimately increase or decrease the likelihood of specific behaviors (Bergquist and Ludwig, 2008; Li and Kim, 2008; Ludwig et al., 2002; Ogren et al., 2010; Pantaleo et al., 2010).

In *C. elegans* and mammals, neuropeptides both positively and negatively regulate synaptic transmission. In *C. elegans*, mutations in enzymes crucial for the processing of neuropeptide precursors into their active forms (*proprotein convertase/egl-3* and *carboxypeptidase/egl-21*), or mutations in proteins required for neuropeptide release (*CAPS/unc-31* and *pkc-1*), cause changes in acetylcholine release from the neuromuscular junction (NMJ)(Jacob and Kaplan, 2003; Sieburth et al., 2005; Sieburth et al., 2007). RNAi knock-down of the neuropeptides *ins-22* and *nlp-21* also decreases cholinergic signaling at the NMJ (Sieburth et al., 2005), demonstrating these peptides positively regulate synaptic transmission. Conversely, knock-down of the neuropeptide receptor *npr-1*, a neuropeptide-Y receptor homolog, or Y54E2A.1, a Galanin receptor homolog, increases signaling at the NMJ (Vashlishan et al., 2008). Direct application of neuropeptides to pharyngeal muscle has also identified positive and negative regulators of muscle depolarization (Rogers et al., 2001). In mammals, neuropeptides can also positively or negatively regulate synaptic transmission. For example, substance P is a potent stimulator of muscle contraction and is pro-convulsant, while neuropeptide-Y inhibits synaptic transmission in mossy fibers and has anti-convulsive properties (Nadler et al., 2007; Pantaleo et al., 2010). Interestingly, the neuropeptide galanin can be either stimulatory or inhibitory, depending on its down-stream

targets (Ogren et al., 2010). Galanin binding to galanin receptor 2 leads to intracellular calcium mobilization, but galanin receptors 1 and 3 have hyperpolarizing actions (Ogren et al., 2010).

A wide array of sensory systems and behaviors are regulated by neuropeptides in both *C. elegans* and mammals. Locomotion, nose-touch response, odor detection, osmolarity detection, foraging behavior, avoidance behaviors, egg-laying, and entry into the dauer larval stage are just a short list of the behaviors that have been shown to be regulated by neuropeptides in *C. elegans* (Li, 2006; Chalasani, 2010; Harris, 2010; Rogers, 2003; Wittenburg, 1999). Mutations in neuropeptides and their receptors can cause subtle changes in feeding and foraging behaviors in *C. elegans*. A single point mutation in the *npr-1* gene can alter *C. elegans* feeding behavior from "solitary" foraging (characterized by slow movement on bacterial lawns and dispersal away from other worms) to "social" foraging (characterized by rapid movement across bacterial lawns and aggregation with other worms)(de Bono and Bargmann, 1998). In response to food removal, an odor-OFF neuron called AWC is activated to initiate turning behaviors that allow the animal to search for new food sources (Chalasani et al., 2007). Execution of these behaviors is regulated by two peptides: one released from AWC (NLP-1) and one that is released from AWC's down-stream targets and provides feed-back inhibition onto AWC (INS-1) (Chalasani et al., 2010).

In mammals, neuropeptides have been shown to modulate long-term potentiation (LTP), and may have stimulatory or inhibitory effects depending on the concentration of neuropeptide and its site of action (Ogren et al., 2010). Learning and memory, pain perception, feeding behavior, sleeping and arousal, drug-seeking, and maternal behaviors are all under the control of neuropeptide regulation (Nadler et al., 2007; Ogren et al., 2010; Pantaleo et al., 2010; Schwarzer, 2009). Neuropeptides and their receptors have been implicated as critical to the pathology of anxiety and depression, drug abuse, neuropathic

pain, epilepsy, and neurodegeneration (Holmes et al., 2003; Nadler et al., 2007; Ogren et al., 2010; Pantaleo et al., 2010; Schwarzer, 2009). Because neuropeptide signaling modulates rather than directly stimulates synaptic transmission, and because neuropeptides and their receptors generally have more restricted anatomical distribution than the classical neurotransmitters, therapies which target neuropeptide signaling pathways may produce fewer side-effects than those which target the classical neurotransmitter systems (Holmes et al., 2003). Thus, neuropeptide signaling pathways represent a promising new therapeutic target for a wide array of neurological and psychiatric diseases.

DCV biosynthesis and trafficking

Neuropeptides are packaged into DCVs at the Golgi as propeptides that are processed by proteases. Multiple bioactive peptides can result from the cleavage of a single propeptide (Li and Kim, 2008; Mains and Eipper, 1999). In *C. elegans*, neuropeptide processing enzymes include the proprotein convertases EGL-3, AEX-5, KPC-1, and BLI-4 and the carboxypeptidase EGL-21 (Li and Kim, 2008). Proprotein convertases are endopeptidases that cleave propeptides at di-basic residues, while the carboxypeptidase removes C terminal amino acids (Mains and Eipper, 1999).

DCVs bud from the trans-Golgi network as immature vesicles that undergo a maturation process which depends on clatherin-dependent segregation into more mature vesicles (Tooze, 1998). This maturation process requires mechanisms to separate peptides and concentrate them in the appropriate vesicles. Peptides can be sorted into a DCV by interaction with a peptide receptor. A neuropeptide processing protease, carboxypeptidase E (CPE), may also function in part to cluster neuropeptides into DCVs (Park and Loh, 2008). In addition, granin proteins within the lumen of the DCV, including chromogranin A and B, may facilitate the granulation of peptides into a “dense-core” that is subsequently separated

into a vesicle (Park and Loh, 2008). A low pH environment also contributes to the granulation of peptides as DCVs form (Park and Loh, 2008). Rab2/UNC-108 is essential for the sorting of peptides in *C. elegans*. In the absence of *rab-2/unc-108*, small peptides cleaved from the *nlp-21* propeptide are not sorted into DCVs, but instead mis-sort into an endosomal compartment (Edwards et al., 2009; Sumakovic et al., 2009).

Once DCVs have budded from the trans-Golgi, they are trafficked to release sites. In worms, flies, and mammals, Kif1A/UNC-104 mediates the trafficking of DCVs down axons (Barkus et al., 2008; Jacob and Kaplan, 2003; Lo et al., 2011; Pack-Chung et al., 2007; Schinkmann and Li, 1994; Zahn et al., 2004). In mammalian hippocampal neurons, Kif1A co-migrates with DCV markers and Kif1A knock-down decreases anterograde DCV velocity (Lo et al., 2011). Likewise, *Drosophila imac/unc-104* is required for transport of DCVs to presynaptic sites (Barkus et al., 2008; Pack-Chung et al., 2007). *Imac/unc-104* mutants have decreased numbers of anterogradely and retrogradely moving DCVs, as well as decreased anterograde DCV velocity (Barkus et al., 2008). In *C. elegans unc-104(e1265)* loss-of-function mutants, DCVs which would normally localize to presynaptic sites accumulate in neuronal cell bodies (Jacob and Kaplan, 2003; Sieburth et al., 2005). In *unc-104* mutants, the velocity of DCV movement is decreased in both the anterograde and retrograde direction in the axon (Zahn et al., 2004). UNC-116/Kif5 has also been implicated in DCV trafficking, because FMRFamide peptides accumulate in *unc-116* mutants (Schinkmann and Li, 1994). In mammals, two other motors have been implicated in DCV trafficking. Trafficking of BDNF-containing DCVs requires Kif5 (Colin et al., 2008) and transportation of insulin-containing DCVs in pancreatic beta cells requires both Kif5 and dynein (Varadi et al., 2003). Myosin Va regulates the number of stationary DCVs in axons and their retrograde velocity (Bittins et al., 2010). Myosin Va has therefore been proposed

to be necessary for re-loading of DCVs onto microtubules to initiate retrograde trafficking following pausing.

Multiple adaptor proteins have been implicated in DCV trafficking. The cytoplasmic tail of CPE is hypothesized to link DCVs to motors (Park et al., 2008a). Immunoprecipitation of CPE pulls down kinesin-2, kinesin-3, and dynein (Park et al., 2008b). Overexpression of the cytoplasmic tail of CPE reduces DCV movements in axons and dendrites, suggesting CPE's tail may be essential for linking DCVs to motors (Park et al., 2008b). The dynactin complex is also required for the bi-directional movement of DCVs in axons and dendrites (Kwinter et al., 2009). Huntingtin (htt), the endogenous protein that is mutated in Huntington's Disease patients, is another important regulator of DCV movement (Caviston and Holzbaur, 2009). Knock down of *htt* decreases DCV movement bi-directionally and *htt* overexpression increases DCV trafficking in both directions (Caviston and Holzbaur, 2009). Interestingly, the phosphorylation status of Htt is a critical regulator of the direction of DCV transport. A phospho-mimetic Htt protein increases anterograde trafficking, while an unphosphorylatable version of Htt favors retrograde trafficking (Colin et al., 2008).

To date, studies of DCV trafficking have not clearly identified different transport mechanisms at work in the axon and dendrite, although some studies suggest that trafficking may be regulated differently in the two different compartments. DCVs can be released from both axons and dendrites and have been observed to move bi-directionally in both subcellular compartments in *C. elegans* and mammals (Bergquist and Ludwig, 2008; de Wit et al., 2006; Kwinter et al., 2009; Vila-Porcile et al., 2009; Zahn et al., 2004). Analysis of DCV movement in axons and dendrites shows that trafficking dynamics differ between the two subcellular compartments. DCVs tend to have slower trafficking, more pausing and more reversals in dendrites, while DCV movement is faster and characterized by longer run lengths in the axon (de Wit et al., 2006; Kwinter et al., 2009). Additionally,

axonal transport of Sema3A-containing DCVs can be regulated by changes in neuronal activity, while dendritic transport is not (de Wit et al., 2006). While many lines of evidence support the role of UNC-104 as the axonal anterograde DCV motor in *C. elegans* (Jacob and Kaplan, 2003; Sieburth et al., 2005; Zahn et al., 2004), analysis of DCV movement in dendritic processes in *unc-104* mutants suggests that UNC-104 is not the motor responsible for dendritic transport in *C. elegans* (Zahn et al., 2004). It is not clear if Kif5 or dynein transport DCVs in dendrites, because studies examining the role of Kif5 and dynein in DCV movement have either used non-neuronal cells (Varadi et al., 2003) or have not analyzed movement in axons and dendrites separately (Colin et al., 2008; Dompierre et al., 2007). As a result, very little is known about how dendritic trafficking of DCVs is regulated.

Regulation of axonal and dendritic DCV release

DCV release shares many features in common with synaptic vesicle release, but there are some aspects of release which are specific for DCVs. Synaptic vesicle and DCV release is activity-dependent and calcium-dependent, but in general synaptic vesicles are released in response to single action potentials, while DCVs are released in response to trains of multiple action potentials (Mains and Eipper, 1999). Synaptic vesicles are released from active zones, where voltage-gated calcium channels are closely localized to synaptic vesicles and their release proteins. DCV docking sites, on the other hand, are located farther from calcium channels, resulting in less sensitive release (Karhunen et al., 2001). In *C. elegans,* DCVs dock at the plasma membrane throughout the axon and are excluded from active zones (Hammarlund et al., 2008).

Synaptic vesicles and DCVs utilize some of the same proteins for release; however some molecules are specific for DCV release. Two Rab proteins, Rab3 and Rab27, have both been shown to regulate both SV release and DCV release (Ch'ng et al., 2008; Fukuda,

2003; Mahoney et al., 2006; Yu et al., 2008). The syntaxin-binding protein UNC-13 promotes docking of both synaptic vesicles and DCVs (Gracheva et al., 2006; Hammarlund et al., 2008; Madison et al., 2005; Weimer et al., 2006) and the syntaxin-interacting protein tomosyn is a negative regulator of both SV and DCV release (Gracheva et al., 2006; Gracheva et al., 2007; McEwen et al., 2006). On the other hand, the calcium-activated protein for secretion (CAPS/UNC-31) is a SNARE-binding protein that is specifically required for DCV docking, but not SV docking (Hammarlund et al., 2008; Speese et al., 2007). In *C. elegans*, protein kinase C (PKC), specifically regulates DCV release but not SV release (Sieburth et al., 2007).

DCVs also differ from synaptic vesicles in that their release does not always occur at synaptic termini in axons. Oxytocin, vasopressin, galanin, and BDNF are released from DCVs in dendrites (Bergquist and Ludwig, 2008; Matsuda et al., 2009; Vila-Porcile et al., 2009). Moreover, the stimuli that trigger DCV release in the dendrite differ from those that trigger axonal release (Bergquist and Ludwig, 2008; Matsuda et al., 2009). Dendritic release of oxytocin occurs in response to release of intracellular calcium stores, does not require action potentials, and can be self-potentiating, while axonal release requires action potentials and extracellular calcium entry through P/Q-type calcium channels, and is not self-potentiating (Bergquist and Ludwig, 2008; Ludwig et al., 2002). Secretion of BDNF from dendrites can be triggered by short (1 min) theta-burst stimulation (TBS), but longer (3 min) stimulation is required to achieve axonal release of BDNF (Matsuda et al., 2009). Moreover, brief spiking activity induces “full collapse” of BDNF-containing DCVs in the dendrite, but results in partial vesicle-fusion in the axon (Matsuda et al., 2009). Therefore, by changing the balance of DCVs trafficked to the dendrite versus axon, a neuron will change the stimuli that cause neuropeptide release.

After neuropeptides have been released, they are not actively re-absorbed into the neuron. Re-uptake channels have been identified for the classical neurotransmitters, but no mechanisms responsible for transporting neuropeptides back into neurons have been identified. The absence of a re-uptake mechanism means that in order to replenish neuropeptides lost after release, the neuron must continuously synthesize and transport new DCVs from the cell body. To keep up with the constant demand for new DCVs, the neuron synthesizes excess DCVs and responds to high DCV demand after increased neuronal activity by regulating DCV trafficking in an activity-dependent manner.

In *Drosophila* motor neurons, excess DCVs traffic in both directions through and between synaptic termini. High activity decreases the amount of retrograde trafficking, allowing the "capture" of extra vesicles as they are needed following release (Shakiryanova et al., 2006). As previously mentioned, activity-dependent regulation of DCV mobility may be an axon-specific phenomenon. The movement of Sema3A-containing DCVs decreases following depolarization and increases following TTX application in the axon, but transport in the dendrite is unaffected by changes in neuronal activity (de Wit et al., 2006).

In summary, release of neuropeptides from DCVs is a key regulator of synaptic transmission and neuronal activity. Because neuropeptides are not recovered after release, trafficking of new DCVs has to be continuous to keep up with demand. While some studies have identified proteins involved in DCV trafficking, little work has been aimed at determining the differences between axonal and dendritic trafficking. Also, almost nothing is known about how DCV trafficking is polarized, an important issue given that DCVs can be released from axons and dendrites. Failure to polarize a DCV could not only result in loss of neuropeptides and neurotrophins at the correct site of release, but could also result in signaling to an inappropriate target. Furthermore, because release from the two different subcellular compartments occurs under different conditions, it might potentially change the

stimuli that cause a DCV to be released. Almost nothing is known about the mechanisms that regulate the polarized trafficking of DCVs. This work describes a novel role for a serine/threonine kinase, cyclin-dependent kinase-5 (CDK-5) in regulation of polarized DCV trafficking.

Cyclin-Dependent Kinase-5

CDK-5 is a proline-directed serine/threonine kinase that is homologous to other cyclin-dependent kinases, but is not activated by cyclin and is not thought to regulate the cell cycle under normal physiological conditions. Instead, CDK-5 is activated by two proteins, p35 and p39, that have a 3-dimensional structure similar to cyclin (Dhariwala and Rajadhyaksha, 2008). p35 can be cleaved by calpain into a protein called p25, which can also activate CDK-5 (Lee et al., 2000).

CDK-5 is expressed fairly ubiquitously, but as p35 and p39 expression are restricted mainly to post-mitotic neurons, CDK-5 activity is highest and best-described in mature neurons (Dhavan and Tsai, 2001). It is now evident that p35 and p39 are also expressed outside of the nervous system as well, and CDK-5 has multiple functions in non-neuronal cells, including pancreatic beta cells, immune cells, muscle cells, and cancer cells (Rosales and Lee, 2006). p35 and p39 are myristolated, which restricts CDK-5's activity to membranes. CDK-5 phosphorylates p35 and p39, which leads to their degradation and allows CDK-5 to regulate its own activity (Dhavan and Tsai, 2001). When p35 is cleaved to p25, it no longer associates with the membrane and allows CDK-5 to phosphorylate different target proteins. Because p25 has a longer half-life than p35, CDK-5 is hyper-activated by p25 (Dhariwala and Rajadhyaksha, 2008). CDK-5 activity can be enhanced by phosphorylation of T15 by Abl kinase. The growth factors BDNF and NGF increase CDK-5 activity by increasing transcription of p35 (Dhariwala and Rajadhyaksha, 2008). Apart from

these two activation mechanisms, little is known about the upstream regulators of CDK-5 activity.

Functions of CDK-5

CDK-5 activity is an important regulator of many aspects of neuronal function, including cellular structure, synapse formation, synaptic transmission, synaptic plasticity, and neurodegeneration.

CDK-5 regulates microtubule dynamics during cellular migration and axon elongation. Phosphorylation of focal adhesion kinase (FAK) is required to pull the nucleus during neuronal migration (Nikolic, 2004). As a result, both *cdk-5* and *p35* knock-out mice have disordered cortical layers, due to a failure of neurons to migrate past each other (Dhavan and Tsai, 2001). CDK-5 also phosphorylates MAP1B to stabilize microtubules during axon outgrowth (Del Rio et al., 2004). Sema3a signaling can recruit p35 to growth cones to regulate axon repulsion. CDK-5 can then phosphorylate collapsin response-mediating protein (CRMP-2) and stabilize the growth cone on one side, which allows axon turning (Uchida et al., 2005).

CDK-5 also regulates synapse formation in mammals and *Drosophila*. *cdk-5* mutants have defects in neuromuscular junction (NMJ) formation and locomotion in *Drosophila* (Kissler et al., 2009). CDK-5 regulates organization of the presynaptic terminal in mammals through regulation of the MAGUK protein CASK (Samuels et al., 2007). It is required for BDNF-dependent dendrite outgrowth and can both positively and negatively regulate spine formation. CDK-5 activity increases spine formation in the nucleus acumbens following drug administration, but also induces spine retraction in hippocampal neurons responding to EphrinA1 signaling (Lai and Ip, 2009).

Many of CDK-5's phosphorylation targets are involved in synaptic transmission. Presynaptically, CDK-5 phosphorylates Munc18 and decreases its association with syntaxin, which promotes binding of syntaxin to other SNAREs and synaptic vesicle release (Lilja et al., 2004). On the other hand, CDK-5 has also been shown to negatively regulate synaptic vesicle release. CDK-5 activity inhibits dopamine release from the striatum (Chergui et al., 2004). In chromaffin cells, CDK-5 inhibits pore formation during vesicle fusion and decreases quantal size (Barclay et al., 2004). Postsynaptically, CDK-5 positively regulates acetylcholine receptor (AChR) transcription in response to neuregulin signaling (Fu et al., 2005), but negatively regulates clustering of AChR at the NMJ. CDK-5 is required for acetylcholine-induced dispersal of AChR from non-synaptic regions of the NMJ (Fu et al., 2005; Yang et al., 2011). Conditional knock-out of *cdk-5* results in increased NMDA receptor subunit NR2B subunit clustering in hippocampal neurons, due to decreased NR2B degradation (Hawasli and Bibb, 2007). In *C. elegans, cdk-5* is required for trafficking of the AMPA-like glutamate receptor GLR-1 to synapses (Juo et al., 2007). CDK-5 also acts as an inhibitor of endocytosis. Phosphorylation of dynamin and amphiphysin by CDK-5 inhibits their interaction with partner proteins and reduces endocytic vesicle formation (Tomizawa et al., 2003).

Given the number of synaptic proteins regulated by CDK-5, it is not surprising that it has been shown to regulate synaptic plasticity. Synaptic plasticity comes in two flavors: potentiation or de-potentiation in response to short-term changes in upstream neuronal activity (long-term potentiation (LTP) and long-term de-potentiation (LTD)) and homeostatic compensations for long-term changes in global activity levels (synaptic homeostasis or scaling). CDK-5 has been shown to play a role in both forms of plasticity, although controversy exists regarding the direction of CDK-5's regulation of LTP and LTD. At presynaptic sites, CDK-5 can mediate synaptic scaling by regulating the size of the resting

pool of synaptic vesicles (Kim and Ryan, 2010). CDK-5 activity increases the size of the resting pool and can maintain synapses in a “silent” state (Kim and Ryan, 2010). In response to decreased neuronal activity, presynaptic CDK-5 levels are decreased to permit vesicles to move from the resting to the releasable pool, allowing silent synapses to be unmasked (Kim and Ryan, 2010). In the postsynaptic cell, CDK-5-dependent phosphorylation of the scaffolding protein SPAR is critical for synaptic homeostasis (Seeburg et al., 2008). CDK-5 phosphorylation primes SPAR for phosphorylation by Plk2, an event which leads to SPAR degradation and is necessary for synaptic homeostasis (Seeburg et al., 2008). Early studies using the CDK-5 inhibitor roscovitine suggested CDK-5 was required for LTP (Lai and Ip, 2009), however these results have been difficult to replicate with roscovitine and other small molecule inhibitors of CDK-5 (Hawasli and Bibb, 2007), potentially because of off-target effects of CDK-5 inhibitors. Conditional knock-out studies of both *cdk-5* and *p35* suggest instead that CDK-5 activity negatively regulates LTP, because both *cdk-5* and *p35* conditional knock-outs have a lowered threshold for LTP induction (Hawasli and Bibb, 2007). *p35* knock-out also impairs long-term depression (LTD) and de-potentiation of LTP (Ohshima et al., 2005).

The consequences of these changes on learning, however, are puzzling. Loss of p35 impairs spatial learning and contextual fear-conditioned memory (Ohshima et al., 2005), while loss of *cdk-5* actually improves spatial memory task performance (Hawasli and Bibb, 2007). Manipulation of CDK-5 activity through over-expression of p25 adds more complexity to the story. Short-term (2 week) expression of p25 enhances LTP and learning, while long-term expression (6 weeks) leads to reduced LTP and learning impairments due to neurodegeneration (Fischer et al., 2005). These differences might be explained by region or neuron-specific effects of CDK-5.

The role of CDK-5 in neurodegeneration

In addition to its wide and varied roles in regulation of normal neuronal function, CDK-5 is also an important player in neurodegenerative disease. Expression of CDK-5 and its activator, p25, are elevated in patients with Alzheimer's Disease (AD) (Cruz and Tsai, 2004; Lee et al., 2000). These two proteins localize to neurofibrillary tangles, which are one of the hallmarks of AD (Takahashi et al., 2000). Cleavage of p35 to p25 can occur in response to multiple forms of cellular stress relevant to neurodegeneration, including excitotoxic glutamate, hypoxic stress, Aβ exposure, and prion protein exposure (Lee et al., 2000; Lopes et al., 2007). Additionally, inhibition of CDK-5 activity has been shown to be neuroprotective in many models of neurodegeneration, including Aβ overexpression (Cruz and Tsai, 2004; Lopes et al., 2007), prion protein overexpression (Lopes et al., 2007), and mutant Huntingtin overexpression (Paoletti et al., 2008). The role of CDK-5 in neurodegeneration appears to be conserved in invertebrates, because p35 overexpression leads to neuronal death and CDK-5 kinase activity is necessary for Aβ-induced neuronal death in *Drosophila* (Lin et al., 2007).

Multiple CDK-5 targets have been implicated in neurodegenerative disease. The best characterized mechanism is p25/CDK-5-induced hyperphosphorylation of the axonal MAP tau (Fischer et al., 2005; Patrick et al., 1999). Hyperphosphorylation of tau by p25/CDK-5 can induce neurofibrillary tangle formation (Cruz and Tsai, 2004). Another CDK-5 substrate, CRMP-2, is also a component of neurofibrillary tangles and elevated levels of phosphoCRMP-2 are found in the brains of AD patients and AD model triple transgenetic mice (Cole et al., 2007; Uchida et al., 2005).

Aberrant CDK-5 activity is also hypothesized to cause neurodegeneration through changes in the cell cycle. p25/CDK-5 can inhibit histone deacetylase 1, which leads to aberrant cell cycle entry, DNA breakage and neuronal death (Kim et al., 2008).

Transcriptional changes may also be important for CDK-5-dependent neurodegeneration. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment leads to dopaminergic neuron death in the substantia nigra and can be used to model Parkinson's disease. MPTP-induced death of dopaminergic neurons occurs through conversion of p35 to p25 and phosphorylation of the transcription factor MEF2 by CDK-5 (Smith et al., 2006). CDK-5 also up-regulates the expression of the one of the APP processing enzymes responsible for APP conversion to Aβ, BACE-1 (Wen et al., 2008). Finally, CDK-5 can directly phosphorylate APP itself at T668 (Cruz and Tsai, 2004). Mutation of T668 to alanine reduces Aβ formation, suggesting CDK-5 dependent phosphorylation at this site could cause more Aβ production and set a positive feed-back loop of increased Aβ processing followed by increased p25 generation (Cruz and Tsai, 2004).

CDK-5 may also regulate neurodegenerative disease through its regulation of axonal transport. Fast axonal transport is critical to supply the presynaptic terminal with new proteins from the cell body and failure to do so can result in synapse loss and axon retraction. Compromised axonal transport has been implicated in AD, Huntington's Disease, Parkinson's Disease, and amyotrophic lateral sclerosis (ALS)(De Vos et al., 2008). Disrupted transportation of DCVs in particular may contribute to neurodegeneration in Huntington's Disease. Neurons expressing mutant forms of *huntingtin* fail to deliver BDNF-containing DCVs to release sites and therefore lack the neurotrophic signals required for cell survival (Gauthier et al., 2004). Mutations in proteins of the dynactin complex, which has been shown to bi-directionally regulate DCV trafficking (Kwinter et al., 2009), have been linked to a slow progressive motor neuron degenerative disease in humans (Puls et al., 2003). Inhibition of CDK-5 activity decreases anterograde transport of axonal membrane-bound organelles by indirectly affecting Kif5/kinesin-1 phosphorylation (Morfini et al., 2004). Recently, CDK-5 was shown to act in parallel with another cyclin-dependent kinase to inhibit

dynein-mediated retrograde trafficking of synaptic vesicles in axons (Ou et al., 2010). By better understanding CDK-5's role in synaptic protein trafficking, we can shed light on its role in neurodegeneration.

Studying synaptic protein trafficking and synaptic function in *C. elegans*

The genetic model organism *C. elegans* provides a useful tool for the study of CDK-5 function in regulation of synaptic protein trafficking. In mammals, *cdk-5* deletion is embryonic lethal, but *cdk-5* null mutant worms are viable (Juo et al., 2007). Because *C. elegans* are transparent, fluorescently tagged proteins of interest can be imaged in live, intact animals rather than in dissociated neuron cultures. This technique is especially useful for the study of organelle and vesicle trafficking, because individual trafficking events can be observed using time-lapse microscopy *in vivo.* The *C. elegans* nervous system is composed of 302 neurons, including sensory neurons, interneurons, and motor neurons. Electronmicroscopic reconstruction of the entire worm was used by White (1986) to map the positions of neuronal cell bodies, neuronal processes, and connectivity of the *C. elegans* nervous system. A subset of *C. elegans* motor neurons, called the DA and DB type motor neurons, are particularly useful for the study of polarized trafficking in neurons. DA and DB neurons have a simple polarized morphology with a dendritic process that receives synaptic inputs from interneurons on the ventral side of the body and an axonal process on the dorsal side of the body that has cholinergic outputs onto muscles (White and Durbin, 1988). This ventral dendrite/dorsal axon polarization allows for easy identification of changes in polarized trafficking of neuronal cargoes. Additionally, simple behavioral assays can be performed on *C. elegans* to test for changes in synaptic activity (Miller et al., 1996a; Nguyen et al., 1995). By assessing an animal's resistance to the inhibitor of acetylcholine esterase aldicarb, we can indirectly measure the amount of cholinergic transmission in *C. elegans.*

In this thesis, I use *C. elegans* to explore the role that CDK-5 plays in regulation of synaptic protein trafficking. Specifically, we find that CDK-5 is required for the polarized trafficking of neuropeptide-containing DCVs. We investigate the mechanisms by which CDK-5 regulates DCV abundance in both the dendrite and the axon. We also explore the role that CDK-5 plays in regulation of DCV and synaptic vesicle release.

CHAPTER 2

Cyclin-dependent kinase-5 regulates the polarized trafficking of dense-core vesicles in *C. elegans* motor neurons

The following chapter represents a manuscript that has been submitted for publication

Cyclin-dependent kinase-5 regulates the polarized trafficking of dense-core vesicles in *C. elegans* motor neurons

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Introduction

Neurons are highly polarized cells with molecularly and functionally distinct dendritic and axonal compartments that ensure the directional flow of information. The establishment and maintenance of neuronal polarity requires strict mechanisms for the polarized sorting of pre- and postsynaptic proteins to axons and dendrites, respectively. Studies of polarized protein trafficking have largely focused on transmembrane proteins in cultured neurons and have found several mechanisms for compartment-specific localization. Some dendritic and axonal transmembrane proteins are directly targeted to their respective destination compartments (Burack et al., 2000; Gu et al., 2003; Gu et al., 2006; Stowell and Craig, 1999). In contrast, other membrane proteins are globally transported to both axonal and dendritic compartments and are then selectively removed from one of the compartments (Bel et al., 2009; Garrido et al., 2001; Leterrier et al., 2006; Sampo et al., 2003; Wisco et al., 2003). Alternatively, dendritic transmembrane proteins can be prevented from entering the axon by an actin and myosin-based selectivity filter (Lewis et al., 2009; Song et al., 2009). Similar selective and global trafficking mechanisms have been described in *C. elegans*. For example, the odorant receptor ODR-10 is selectively targeted to cilia at the ends of *C. elegans* sensory neuron dendrites (Dwyer et al., 2001), whereas other postsynaptic membrane proteins are trafficked to both axons and dendrites in interneurons and then selectively removed from the axonal compartment membrane (Margeta et al., 2009).

Much less is known about the polarized trafficking mechanisms by which neurons target secreted cargo, like neuropeptide-filled dense-core vesicles (DCVs), to axons and dendrites. DCVs are packaged with their protein cargo at the *trans*-golgi network in the cell body and must be continuously and efficiently transported to their sites of release. In *C. elegans* and *Drosophila*, DCVs are transported from the cell body to axons by the microtubule plus-end directed kinesin, UNC-104/KIF1A (Barkus et al., 2008; Jacob and

Kaplan, 2003; Pack-Chung et al., 2007; Sieburth et al., 2005; Zahn et al., 2004). DCVs contain diverse cargo such as neuropeptides, neurotrophins, and peptide hormones. The regulated release of DCVs can modulate synaptic transmission, synaptic plasticity, and behavior (Burgoyne and Morgan, 2003; Lessmann and Brigadski, 2009; Li and Kim, 2008; Nassel, 2002; Park and Loh, 2008; Samson and Medcalf, 2006). Similarly, neuropeptides can modulate classical neurotransmitter activity and alter several behaviors in *C. elegans* (Edwards et al., 2009; Husson et al., 2007; Jacob and Kaplan, 2003; Kass et al., 2001; Liu et al., 2007; Nelson et al., 1998; Ringstad and Horvitz, 2008; Rogers et al., 2001; Sumakovic et al., 2009).

DCVs in both neurosecretory cells and neurons undergo a complex biogenesis and maturation process resulting in the generation of DCVs carrying unique and diverse cargo (Borgonovo et al., 2006; Dikeakos and Reudelhuber, 2007; Edwards et al., 2009; Sumakovic et al., 2009). These different populations of DCVs are transported from the cell body to distinct compartments of the neuron for secretion (i.e. presynaptic vs postsynaptic compartments). For example, DCVs containing brain-derived neurotrophic factor are trafficked, likely via different mechanisms, to both axons and dendrites and can be specifically targeted to pre- and postsynaptic sites for release (Lessmann and Brigadski, 2009). Studies in *Aplysia* bag cell neurons showed that multiple neuropeptides, such as the Bag Cell Peptides and Egg-Laying Hormone peptide, are generated from a common precursor, but sort into distinct DCV populations that are then differentially trafficked to unique subcellular locations (Fisher et al., 1988). Similarly, the neuropeptide vasopressin is predominantly targeted to nerve terminals, whereas the neuropeptide galanin is targeted to dendrites in rat hypothalamic neurons (Landry et al., 2003). Thus, although distinct populations of DCVs can be targeted to their sites of release in different subcellular

compartments, much remains to be learned about the specific molecules involved in polarized DCV trafficking in neurons.

We used the transparent nematode *C. elegans* to study the polarized trafficking of neuropeptide-containing dense-core vesicles to axons and dendrites of cholinergic motor neurons *in vivo*. We show here that cyclin-dependent kinase-5 (CDK-5) and its activator CDKA-1/p35 are required to maintain the normal polarized distribution of DCVs in motor neurons. We also show that mutations in *syd-2*/Liprin-α or kinesin *unc-104*/KIF1A result in defects in polarized DCV trafficking. However, genetic double mutant analyses indicate that *syd-2* and *unc-104* are not required for the increased accumulation of DCVs in *cdk-5* mutant dendrites. Instead we find that cytoplasmic dynein is required, because mutations in dynein heavy chain, *dhc-1*, completely block the increased accumulation of DCVs in *cdk-5* mutant dendrites. Our data are consistent with a model where CDK-5 inhibits trafficking of DCVs into dendrites.

Results

CDK-5 regulates the polarized distribution of dense core vesicles in motor neurons

We studied the polarized trafficking of neuropeptide-containing dense-core vesicles (DCVs) in DA and DB cholinergic motor neurons in *C. elegans*. Each DA/DB neuron has a simple polarized morphology consisting of a cell body and a single dendrite in the ventral nerve cord (VNC), where it receives inputs, and a single axon in the dorsal nerve cord (DNC), where it makes synapses onto muscle and GABAergic motor neurons (Fig. 2-1 A)(White et al., 1986). We visualized DCVs in a subset of DA/DB motor neurons by expressing a Venus-tagged neuropeptide, insulin-like protein 22 (INS-22::Venus) under the control of the *Punc-129* promoter (Sieburth et al., 2005; Sieburth et al., 2007). We chose the

CHAPTER 2

FIGURE 1

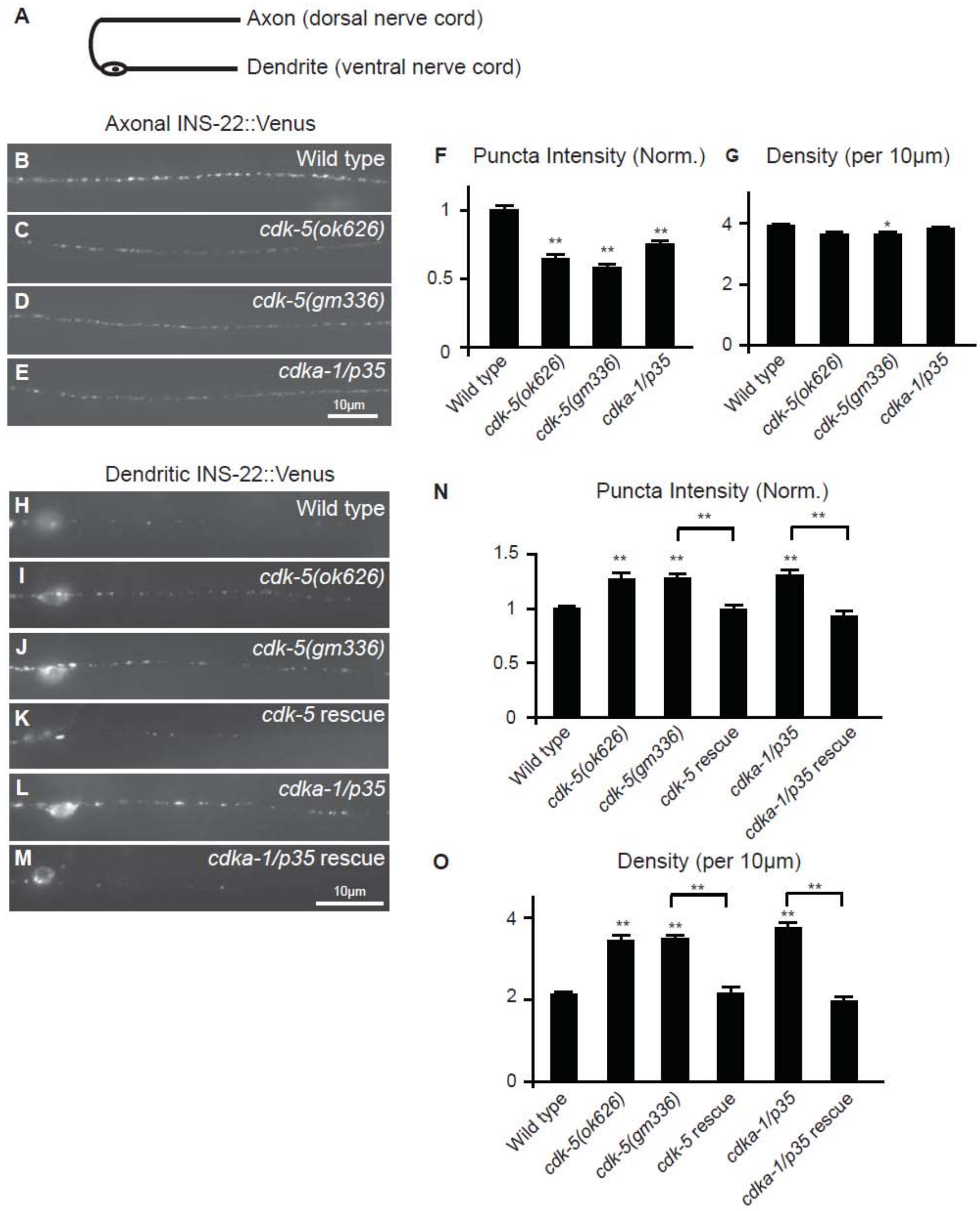


Figure 2-1. CDK-5 regulates the polarized distribution of DCVs in DA/DB motor neurons. (A) Schematic diagram of a DA/DB motor neuron showing the axon in the DNC and dendrite in the VNC. (B-E) Representative images of INS-22:Venus in axons of young adult wild-type (B), *cdk-5(ok626)*(C), *cdk-5(gm336)*(D), and *cdka-1(gm335)*(E) animals. (F-G) Quantification of INS-22::Venus puncta fluorescence intensity (F) and density (G) in the axon for wild-type (n=70), *cdk-5(ok626)* (n=29), *cdk-5(gm336)* (n=40), and *cdka-1/p35* (n=44) animals. (H-M) Representative images of INS-22::Venus in dendrites of young adult wild-type (H), *cdk-5(ok626)* (I), *cdk-5(gm336)* (J), and *cdka-1(gm335)* (L) animals. (K) Rescue of *cdk-5(gm336)* by expression of wild-type *cdk-5* cDNA under the control of the DA/DB motor neuron-specific promoter, *Punc-129*. (M) Rescue of *cdka-1(gm335)* by expression of wild-type *cdka-1/p35* cDNA under the control of the *unc-129* promoter. (N-O) Quantification of INS-22::Venus puncta fluorescence intensity (N) and density (O) in the dendrite of wild-type (n=132), *cdk-5(ok626)* (n=31) *cdk-5(gm336)*(n=99), *Punc129::cdk-5;cdk-5(gm336)*(n=21), *cdka-1/p35* (n= 39), and *Punc129::cdka-1;cdka-1(gm335)*(n=20) animals. For this and all subsequent figures, error bars show standard error from the mean (SEM). Unless noted otherwise, values that differ significantly from wild type (Student's *t* test) are indicated on graphs (* $p \leq 0.01$, ** $p \leq 0.001$) and values that do not differ ($p > 0.05$) are denoted by n.s.

neuropeptide *ins-22* because it is expressed in ventral cord motor neurons and has been implicated in regulation of synaptic transmission at the *C. elegans* NMJ (Pierce et al., 2001; Sieburth et al., 2005). In addition, INS-22::Venus has been well characterized as a DCV marker (Edwards et al., 2009; Sieburth et al., 2005; Sieburth et al., 2007). In wild-type animals, INS-22::Venus has a highly polarized subcellular distribution where it is largely excluded from the motor neuron dendrites and is localized in a punctate manner in the motor neuron axons (Fig. 2-1 B)(Sieburth et al., 2007). INS-22::Venus co-localizes with and adjacent to Synaptobrevin in the axon (Sieburth et al., 2007), consistent with electron microscopy findings which indicate that DCVs are broadly distributed at presynaptic sites and only slightly enriched at the active zone (Hammarlund et al., 2008). Trafficking of INS-22::Venus to the axon requires the anterograde kinesin motor UNC-104/KIF1A, as has been shown for several other DCV markers and endogenous neuropeptides (Jacob and Kaplan, 2003; Sieburth et al., 2005; Sieburth et al., 2007; Zahn et al., 2004).

CDK-5 and its cyclin-like activator CDKA-1/p35 are required for diverse cellular functions including synaptic transmission and plasticity (Cheung et al., 2006; Dhavan and Tsai, 2001). In particular, CDK-5 regulates the anterograde transport of membrane-bound organelles in the squid giant axon (Morfini et al., 2004; Ratner et al., 1998). To investigate whether CDK-5 contributes to the transport of axonal cargoes in *C. elegans*, we analyzed the distribution of INS-22::Venus-containing DCVs in DA/DB motor neurons in *cdk-5(gm336)* and *cdk-5*(*ok626*) mutant animals using quantitative fluorescence microscopy. The *gm336* allele and the *ok626* allele are both predicted null mutations (Juo et al., 2007). We imaged INS-22::Venus fluorescence in the axons of the DA/DB motor neurons in a posterior section of the DNC and quantified the density and fluorescence intensities of INS-22::Venus puncta using custom written software (see Materials and methods, (Burbea et al., 2002)). INS-22::Venus puncta fluorescence intensities were decreased by about 40% ($p < 0.001$) in the

motor neuron axons of *cdk-5(gm336)* and *cdk-5(ok626)* mutant animals compared to wild-type controls (Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *cdk-5(ok626)*: 0.64±0.03; $p<0.001$; *cdk-5(gm336)*: 0.58±0.02; $p<0.001$)(Fig. 2-1 B-D, F). We also analyzed the distribution of INS-22::Venus in the motor neuron axons of animals with loss-of-function mutations in CDK-5's activator, CDKA-1/p35. We found that INS-22::Venus puncta fluorescence intensities decreased by about 25% ($p<0.001$) in *cdka-1*(*gm335*) loss-of-function mutant animals compared to wild-type controls (Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *cdka-1(gm335)*: 0.75±0.03; $p<0.001$) (Fig. 2-1 B-E). There was no significant change in INS-22::Venus puncta densities in the motor neuron axons of *cdka-1*(*gm335*) or *cdk-5*(*ok626*) mutant animals (Average puncta density (per 10μm)±SEM: Wild type: 3.9±0.06; *cdk-5(ok626)*: 3.61±0.13; $p>0.05$ vs WT; *cdka-1(gm335)*: 3.81±0.06; $p>0.05$ vs WT)(Fig. 2-1 G). The decrease in INS-22::Venus puncta fluorescence intensities in *cdk-5* mutants could be due to either a change in the number of DCVs in each axonal cluster, or a change in the amount of INS-22::Venus neuropeptides packaged in each DCV. We favor the first interpretation based on the analysis of INS-22::Venus fluorescence contained in mobile puncta in wild type and *cdk-5* mutants (described below). These results suggest that the abundance of DCVs in the axons of DA/DB motor neurons is regulated by CDK-5 and its activator CDKA-1/p35.

To determine if the loss of INS-22::Venus fluorescence at presynaptic sites was caused by alterations in DCV trafficking in *cdk-5* mutants, we examined the motor neuron cell bodies and dendrites for DCV accumulation. We found no change in the abundance of INS-22::Venus in the motor neuron cell bodies of *cdk-5(gm336)* mutants compared to controls (INS-22::Venus fluorescence (Norm.)±SEM: WT:1.00±0.04; *cdk-5*: 1.10±0.05, $p>0.05$)(Fig. 2-5 G-H, K), however we found a significant increase in INS-22::Venus puncta fluorescence intensity and density in the motor neuron dendrites of *cdk-5(gm336)*, *cdk-*

5(ok626), and *cdka-1(gm335)* mutants compared to wild-type animals (Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *cdk-5(ok626)*: 1.26±0.07; p<0.001; *cdk-5(gm336)*: 1.28±0.05; p<0.001; *cdka-1(gm335)*: 1.3±0.05, p<0.001)(Average puncta density (per 10μm)±SEM: Wild type: 2.13±0.07; *cdk-5(ok626)*: 3.42±0.14; p<0.001; *cdk-5(gm336)*: 3.47±0.10, p<0.001; *cdka-1(gm335)*: 3.74±0.14; p<0.001)(Fig. 2-1 H-O). The increase in INS-22::Venus puncta fluorescence intensity and density in the dendrites of *cdk-5* mutant animals can be rescued by expression of wild-type *cdk-5* cDNA under the control of a DA/DB motor neuron-specific promoter, *Punc129 (cdk-5* rescue)(Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *cdk-5(gm336)*: 1.28±0.05; *cdk-5* rescue: 0.98±0.05; p<0.001 vs *cdk-5*)(Average puncta density (per 10 μm)±SEM: Wild type: 2.13±0.07; *cdk-5(gm336)*: 3.47±0.10; *cdk-5* rescue: 2.15±0.18; p<0.001 vs *cdk-5*)(Fig. 2-1 J-K, N-O)(Sieburth et al., 2005). Likewise, the increase in INS-22::Venus puncta fluorescence intensity and density in the motor neuron dendrites of *cdka-1* mutant animals can be rescued by expression of wild-type *cdka-1* cDNA under the control of the *unc-129* promoter (*cdka-1* rescue)(Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *cdka-1(gm335)*: 1.3±0.05; *cdka-1* rescue: 0.92±0.06; p<0.001 vs *cdka-1*)(Average puncta density (per 10 μm)±SEM: Wild type: 2.13±0.07; *cdka-1(gm335)*: 3.74±0.14; *cdka-1* rescue: 1.96±0.12; p<0.001 vs *cdka-1*)(Fig. 2-1 L-O). Rescue of the decrease in INS-22::Venus puncta fluorescence in the axons of these mutants was partial (Fig. 2-S1 A-J), perhaps due to incomplete rescuing activity of these transgenes. Expression of wild-type *cdk-5* cDNA under the control of a cholinergic neuron-specific promoter (*cdk-5* wt rescue) also rescued the increased accumulation of INS-22::Venus in the dendrites of *cdk-5*(*gm336*) mutants, whereas a kinase-dead version of *cdk-5* (*cdk-5* k.d. rescue) did not rescue (Fig. 2-S1 K-P). In addition, analysis of another DCV marker, a GFP-tagged version of the membrane protein

FIGURE S1

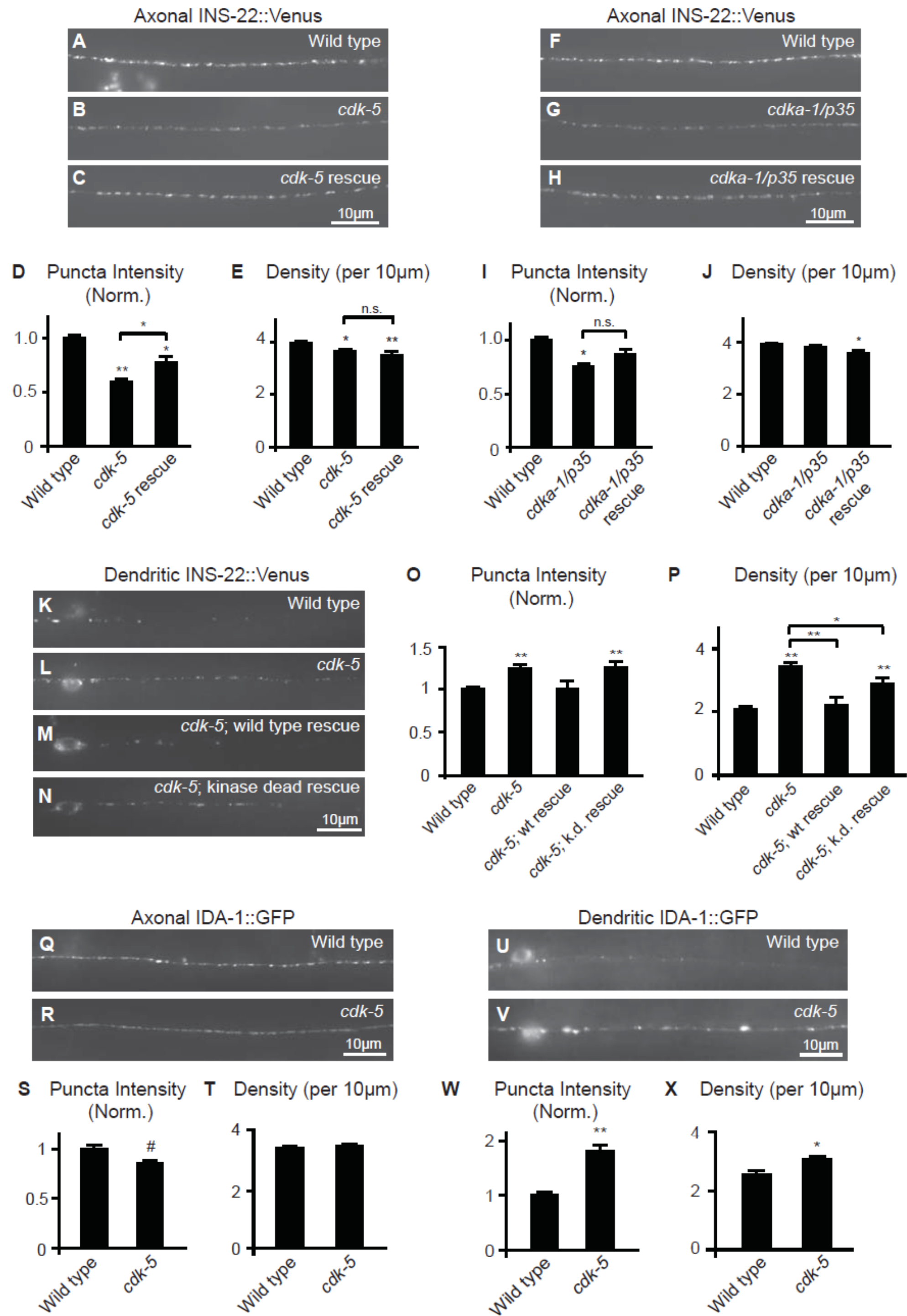


Figure 2-S1. DA/DB motor neuron-specific expression of *cdk-5* and *cdka-1/p35* partially rescues axonal decreases in DCV markers in *cdk-5* and *cdka-1/p35* mutants. (A-J) Representative images of INS-22::Venus puncta in axons of young adult wild-type (A) *cdk-5(gm336)* (B) and *Punc129::cdk-5;cdk-5(gm336)* rescue (C) animals. (D-E) Quantification of axonal INS-22::Venus puncta fluorescence intensity (D) and density (E) for wild type (n=88), *cdk-5* (n=40), and *cdk-5* rescue (n=22). (F-H) Representative images of young adult wild-type (F), *cdka-1(gm335*) (G), and *Punc129::cdka-1; cdka-1(gm335)* rescue (H) animals. (I-J) Quantification of axonal INS-22::Venus puncta fluorescence intensity (I) and density (J) for wild-type (n=70), *cdka-1* (n=44), and *cdka-1/p35* rescue (n=21) animals. **CDK-5's kinase activity is required for regulation of DCV polarity.** (K-N) Representative images of INS-22::Venus puncta in dendrites of young adult wild-type (K), *cdk-5(gm336)* (L), *cdk-5(gm336);Punc17::cdk-5(wt)* rescue (M), and *cdk-5(gm336);Punc17::cdk-5(D144N)* kinase-dead rescue (N) animals. (O-P) Quantification of INS-22::Venus puncta fluorescence intensity (O) and density (P) for wild-type (n=77), *cdk-5(gm336)*(n=63), *cdk-5;* wild-type rescue (n=18), and *cdk-5*;kinase-dead rescue (n=30) animals. **CDK-5 regulates polarized trafficking of DCV membrane proteins** (Q-X) Representative images of IDA-1::GFP puncta in motor neuron axons (Q-R) and dendrites (U-V) of wild type and *cdk-5(gm336)* mutant animals. Quantification of axonal INS-22::Venus puncta fluorescence intensity (S) and density (T) for wild type (n=34), *cdk-5* (n=38). Quantification of dendritic INS-22::Venus puncta fluorescence intensity (W) and density (X) for wild type (n=25), *cdk-5* (n=27). For this and all subsequent Figures, unless otherwise noted, error bars show standard error from the mean (SEM). Values that differ significantly from wild type (Student's *t* test) are denoted above each bar ($^{\#}p<0.05$, * $p \leq 0.01$, ** $p \leq 0.001$).

IDA-1/IA-2 (insulinoma-associated protein 2)(Cai et al., 2004; Edwards et al., 2009; Solimena et al., 1996; Zhou et al., 2007), in wild type and *cdk-5* mutants revealed similar defects in the polarized distribution of DCVs (Fig. 2-S1Q-X). These results suggest that CDK-5 and its activator CDKA-1/p35 function in DA/DB motor neurons to regulate the normal polarized axonal-dendritic distribution of DCVs.

The defect in the polarized distribution of DCVs observed in *cdk-5* mutants could be due to defects in axon outgrowth or presynaptic development. To test this possibility, we analyzed the number and morphology of the DA/DB motor neurons in wild type and *cdk-5* mutants. We found no change in the number of DA/DB motor neurons expressing INS-22::Venus (# cell bodies $\pm$SEM: Wild-type: 10.2$\pm$0.2; *cdk-5(gm336)*: 9.9$\pm$0.2; $p=0.2$) or in the gross morphology of their axonal and dendritic processes (data not shown). In addition, the density of INS-22::Venus puncta in the axons of these motor neurons is largely unchanged in *cdk-5* and *cdka-1* mutant animals compared to controls (Fig. 2-1), suggesting that the number of presynaptic sites is unaltered.

To further test for changes in presynaptic development, we examined the axonal distribution of the presynaptic active zone protein UNC-10/RIM-1. Fluorescently-tagged UNC-10 (under the control of the *Punc-129* promoter) localizes to synapses in the axons of DA/DB motor neurons and is largely absent from the dendrites (Fig. 2-S2 A-F) (Sieburth et al., 2005). We found no significant change in UNC-10::GFP puncta fluorescence intensities in motor neuron axons of *cdk-5(gm336)* mutants compared to wild-type controls (Fig. 2-S2 C). Although there was a small decrease in the density of UNC-10::GFP in *cdk-5* mutant axons ($p<0.05$), there was no change in the distribution of UNC-10::GFP in *cdk-5* mutant dendrites (Fig. 2-S2 D). Analysis of the distribution of the synaptic vesicle (SV) marker RAB-3::Venus in *cdk-5* mutants showed that while there was no change in RAB-3::Venus

FIGURE S2

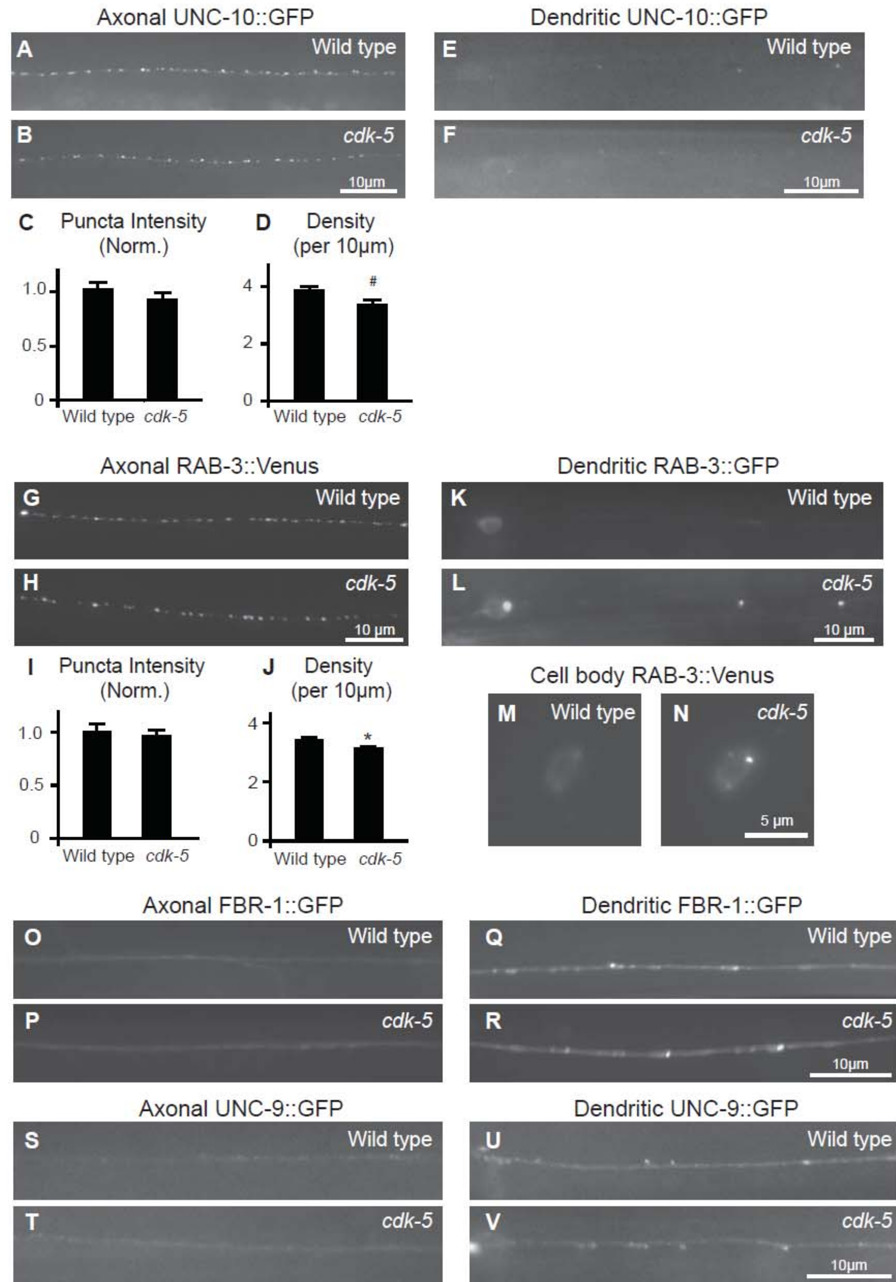


Figure 2-S2. Analysis of axonal and dendritic markers in *cdk-5* mutants

(A-B) Representative images of the presynaptic marker UNC-10::GFP in axons of young adult wild-type (A) and *cdk-5(gm336)* (B) animals. (C-D) Quantification of UNC-10::GFP puncta fluorescence intensity (C) and density (D) in wild-type (n=21) and *cdk-5* (n=18) animals. (E-F) Representative images of UNC-10::GFP in dendrites of wild type (E) and *cdk-5(gm336)* mutants (F). (G-H) Representative images of the synaptic vesicle marker RAB-3::Venus in axons of wild-type (G) and *cdk-5(gm336)* (H) animals. (I-J) Quantification of RAB-3::Venus puncta fluorescence intensity (I) and density (J) in wild-type (n=20) and *cdk-5* (n=24) animals. (K-L) Representative images of RAB-3::GFP localization in dendrites of young adult wild-type (K) and *cdk-5(gm336)* (L) animals. (M-N) Representative images of RAB-3::Venus in cell bodies of wild-type (M) and *cdk-5* (N) mutant animals. (O-R) Representative images showing the dendritic marker Fibrillin::GFP in motor neuron axons (O-P) and dendrites (Q-R) of young adult wild-type (O, Q) and *cdk-5(gm336)* (P, R) mutant animals. (S-V) Representative images showing the dendritic marker UNC-9::GFP in motor neuron axons (S-T) and dendrites (U-V) of young adult wild-type (S, U) and *cdk-5(gm336)* (T, V) mutant animals. Error bars show standard error from the mean (SEM). Values that differ significantly from wild-type (Student's *t* test) are denoted with asterisks (*) above each bar (*$p \leq 0.01$, #$p<0.05$).

puncta intensity in the *cdk-5* mutant axons, there was a small but significant ($p<0.01$) decrease in the density of RAB-3::Venus puncta in axons together with a corresponding increase of RAB-3 markers in the cell body and dendrites of *cdk-5* mutants (Fig. 2-S2 G-N). These data are consistent with a recent study showing that mutations in cyclin-dependent kinases regulates SV trafficking (Ou et al., 2010). Taken together, our results suggest that the defect in the polarized distribution of DCVs observed in *cdk-5* mutants is not due to gross defects in axonal development.

The increased abundance of DCVs in motor neuron dendrites could be the result of a general defect in dendritic trafficking that could, for example, be caused by a loss of dendrite identity. We tested this possibility by analyzing the distribution of two markers that have been shown to localize to the somatodendritic domain of DA/DB motor neurons, Fibrillin::GFP and the invertebrate GAP junction innexin protein UNC-9::GFP (Poon et al., 2008; Sieburth et al., 2005). We found normal somatodendritic localization of both UNC-9::GFP and Fibrillin::GFP in *cdk-5* mutant animals, suggesting that a general defect in trafficking of dendritic proteins is unlikely (Fig. 2-S2 O-V). These results suggest that the DCV trafficking defect observed in *cdk-5* mutants is not likely due to general defects in axon or dendrite development or the establishment and maintenance of axonal-dendritic polarity.

Because CDK-5 can regulate synaptic transmission in other systems (Cheung et al., 2006; Dhavan and Tsai, 2001), and activity can regulate DCV trafficking (Shakiryanova et al., 2006), we tested if the defect in the polarized distribution of DCVs in *cdk-5* mutants was a secondary consequence due to a role for CDK-5 in synaptic transmission. The RAB-3 interacting protein UNC-10/RIM-1 is involved in synaptic vesicle priming and loss-of-function mutations in *unc-10* result in defects in synaptic transmission at the *C. elegans* neuromuscular junction (NMJ)(Koushika et al., 2001; Miller et al., 1996a). We analyzed whether INS-22::Venus-containing DCVs accumulate in motor neuron dendrites of *unc-*

10(e102) mutants. *unc-10(e102)* mutants have stronger defects in synaptic transmission at the NMJ than *cdk-5* mutants based on the aldicarb-paralysis assay (Koushika et al., 2001; Miller et al., 1996a) and (P.R.G. and P.J. unpublished data). In contrast to our results in *cdk-5* mutants, we found no difference in the abundance of INS-22::Venus fluorescence in motor neuron dendrites of *unc-10* mutants (Normalized puncta intensity±SEM: WT: 1.0±0.06; *unc-10*: 0.87±0.07; p=0.61, Puncta density±SEM: WT: 1.74±0.16; *unc-10*: 1.85±0.22; p=0.67). This result suggests that the defect in the polarized distribution of DCVs observed in *cdk-5* mutant animals is not a secondary consequence of a defect in synaptic transmission.

CDK-5 regulates anterograde DCV trafficking to the dendrite

DCVs are highly mobile and move bidirectionally in a saltatory fashion in both axons and dendrites of *C. elegans*, *Drosophila* and mammalian neurons (Barkus et al., 2008; de Jong et al., 2008; Gauthier et al., 2004; Kwinter et al., 2009; Zahn et al., 2004). Similarly, we found that a large fraction of INS-22::Venus puncta move along axons and dendrites. To gain insight into why DCVs accumulate in the motor neuron dendrites of *cdk-5* mutant animals, we performed time-lapse microscopy of mobile INS-22::Venus-containing vesicles in the motor neuron dendrites of wild-type and *cdk-5* mutant young adult animals. Unfortunately, accurate analysis of mobile INS-22::Venus puncta in motor neuron axons is challenging due to the high density of bright presynaptic clusters of DCVs and the curved morphology of the axon commissures. In order to distinguish between DCVs moving in anterograde (away from the cell body) versus retrograde (towards the cell body) directions in the dendrites, we imaged mobile INS-22::Venus puncta in a section of the VNC that only contains the Venus marker in anteriorly-projecting DA motor neuron dendrites (see Materials and methods).

We generated kymographs from our time-lapse imaging data to measure anterograde and retrograde DCV velocities and run lengths. Kymographs represent the distance travelled by a particle as a function of time. In kymographs, stationary DCVs appear as vertical lines and moving DCVs appear as diagonal lines, where the slope of the line can be used to calculate particle velocity. In wild-type animals, we observed bidirectional saltatory movement of DCVs, with anterograde and retrograde velocities reaching more than 3 μm/s and maximum run lengths reaching greater than 20 μm, consistent with previous reports in *C. elegans* and mammals (Kwinter et al., 2009; Zahn et al., 2004). The average anterograde and retrograde DCV velocities in wild-type animals were 1.71±0.13 μm/s and 1.09±0.06 μm/s, respectively, and the average anterograde and retrograde DCV run lengths were 8.28±1.33 μm and 4.17±0.44 μm, respectively (Table 2-1).

Kymograph analysis of INS-22::Venus-containing DCVs in the motor neuron dendrites of *cdk-5(gm336)* mutant animals revealed multiple changes in anterograde DCV trafficking compared to wild-type controls (Fig. 2-2 and Table 2-1). First, we discovered an increase in the number of DCVs moving in the anterograde direction in *cdk-5* mutant dendrites (Average No. of anterograde puncta±SEM: Wild type: 5.7±0.7; *cdk-5*: 9.6±0.7; p<0.001)(Fig. 2-2 C). Second, we found an increase in the number of stationary DCVs in *cdk-5* mutant dendrites (Average No. of stationary puncta±SEM: Wild type: 3.1±0.6; *cdk-5*: 8.3±0.7; p<0.001)(Fig. 2-2 C). Third, we observed a decrease in the average anterograde DCV run length in *cdk-5* mutant dendrites (Average anterograde run length±SEM (μm): Wild-type: 8.28±1.33; *cdk-5*: 4.64±0.41; p<0.05)(Table 2-1). Finally, we found that the average anterograde velocity of these DCVs was decreased in *cdk-5* mutants (Average anterograde velocity±SEM: Wild-type (μm/s): 1.71±0.13; *cdk-5*: 1.40±0.07; p<0.05)(Table 2-1). In contrast, there was no change in the number, velocity, or run length of DCVs moving in the retrograde direction in *cdk-5* mutant dendrites (Fig. 2-2 and Table 2-1).

CHAPTER 2

Table 1

	Direction						Velocity (µm/s)		Run length (µm)	
	anterograde		stationary		retrograde					
	avg. no.	%	avg. no.	%	avg. no.	%	anterograde	retrograde	anterograde	retrograde
wild type	5.7 ± 0.7	33 ± 4%	3.1 ± 0.6	18 ± 4%	8.4 ± 0.7	49 ± 4%	1.71 ± 0.13	1.09 ± 0.06	8.28 ± 1.33	4.17 ± 0.44
cdk-5	9.6 ± 0.7 *	34 ± 2%	8.3 ± 0.7 *	29 ± 3% *	10.2 ± 0.9	36 ± 3% *	1.40 ± 0.07 #	1.00 ± 0.05	4.64 ± 0.41 #	3.66 ± 0.29
syd-2	2.5 ± 0.6 * +	15 ± 3% * +	10.3 ± 1.1 *	63 ± 7% * +	3.5 ± 0.6 * +	22 ± 4% * +	1.41 ± 0.19	0.86 ± 0.06 #	1.78 ± 0.31 * +	1.87 ± 0.27 *
cdk-5;syd-2	5.5 ± 0.7 + °	26 ± 3% + °	10.4 ± 0.8 *	49 ± 4% * +	5.4 ± 0.6 * + §	25 ± 3% * +	1.14 ± 0.10 * †	0.91 ± 0.08	3.29 ± 0.66 * + §	3.00 ± 0.32 °
unc-104	3.2 ± 0.5 * +	19 ± 3% * +	11.9 ± 1.2 * +	67 ± 5% * +	2.3 ± 0.4 * +	14 ± 3% * +	0.90 ± 0.06 * + §	1.00 ± 0.07	4.87 ± 0.93 °	3.35 ± 0.58 §

Table 2-1. INS-22::Venus transport in wild type, *cdk-5*, *syd-2*, and *unc-104* mutant animals. Data were generated from 20 second kymographs of wild type (n=18), *cdk-5 (gm336)* (n=23), *syd-2(ju37)*(n=26), *cdk-5(gm336);syd-2(ju37)* (n=22), and *unc-104(e1265)* (n=17) mutant dendrites. Means ± SEM are reported. P values were calculated using Student's T test for number and percentage of anterograde, retrograde, and stationary puncta, and for puncta velocity. P values for run length were calculated using the Kolmogorov-Smirnov test. P values are denoted by

*$p<0.01$ vs. wild type,

#$p<0.05$ vs. wild type,

+$p<0.01$ vs. *cdk-5,*

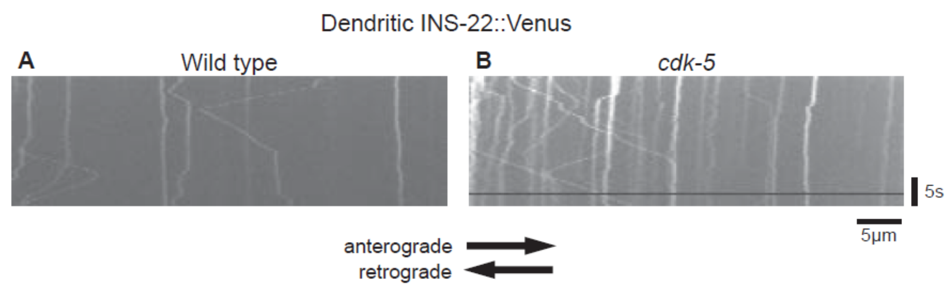
†$p<0.05$ vs. *cdk-5,*

º $p<0.01$ vs. *syd-2,*

§$p<0.05$ vs. *syd-2.*

CHAPTER 2

FIGURE 2



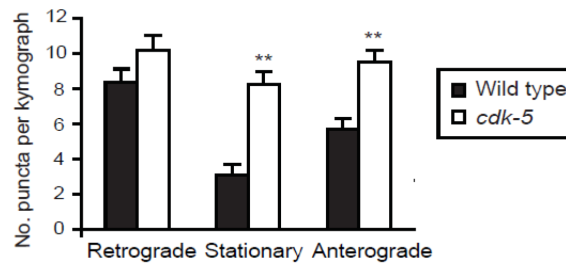


Figure 2-2. CDK-5 regulates anterograde DCV trafficking in motor neuron dendrites. (A-B) Representative kymographs generated from a 20 second movie of INS-22::Venus movement in dendrites of young adult wild-type (A) and *cdk-5(gm336)* mutant (B) animals. (C) Quantification of the number of INS-22::Venus puncta observed moving anterogradely, retrogradely, or remaining stationary as calculated from kymographs of wild-type (n=18) and *cdk-5* (n=21) animals.

These changes in anterograde DCV trafficking in *cdk-5* mutants suggest a potential mechanism to explain the accumulation of DCVs in dendrites. In *cdk-5* mutant dendrites, the increased DCV accumulation (i.e. increased number of stationary DCVs) could result from the fact that there is an increase in the amount of anterograde DCV trafficking without a compensatory increase in the amount of retrograde DCV trafficking. Thus, CDK-5 may negatively regulate DCV loading onto or anterograde trafficking of a dendrite-directed motor. The decreased run length of DCVs moving in the anterograde direction in *cdk-5* mutant dendrites could also contribute to the number of stationary DCVs in the dendrite. For example, stationary DCVs might accumulate in the dendrite if there is increased probability of DCV cargo being released from their motors or of DCV cargo-motor complexes being released from microtubules. Taken together, our data are consistent with a model whereby stationary DCVs accumulate in *cdk-5* mutant dendrites due to both an increase in the number of DCVs moving into the dendrite and a decrease in anterograde DCV run lengths. Because we analyzed DCV movement in young adult animals, our time-lapse data are consistent with the idea that CDK-5 regulates polarized DCV trafficking in fully developed DA/DB motor neurons and is required to maintain the polarized axonal-dendritic distribution of DCVs in the mature nervous system.

As mentioned above, changes in INS-22::Venus puncta fluorescence could represent either changes in the number of DCVs in each cluster or changes in the amount of INS-22::Venus neuropeptides contained in each DCV. To distinguish between these possibilities, we measured the amount of INS-22::Venus fluorescence in mobile versus stationary puncta. Assuming that mobile puncta likely represent single DCVs, this analysis allows us to estimate the amount of INS-22::Venus neuropeptide contained in each DCV in wild type and *cdk-5* mutants. Histogram analysis of INS-22::Venus puncta fluorescence of mobile versus stationary puncta in wild type and *cdk-5* mutants is consistent with the first

prediction (Fig. 2-S3). First, there was no difference in the average INS-22::Venus fluorescence intensity in mobile puncta in the dendrites of wild type and *cdk-5* mutants (Average puncta intensity (Norm.): Wild type: 1.0±0.03, *cdk-5*: 1.0±0.03, p=0.9)(Fig. 2-S3). Second, INS-22::Venus fluorescence intensities of stationary puncta in *cdk-5* mutants shifted to higher values revealing multiple peaks with increased fluorescence intensities compared to the intensities of mobile puncta (Fig. 2-S3B). Assuming that mobile puncta largely represent single DCVs (see Materials and methods), these data are consistent with the idea that changes in INS-22::Venus puncta fluorescence observed in *cdk-5* mutant axons and dendrites are not due to changes in INS-22::Venus packaging, but likely represent changes in the number of DCVs per cluster. Taken together, these results suggest that CDK-5 regulates polarized DCV trafficking in motor neurons.

SYD-2/Liprin-α regulates the polarized distribution of DCVs

SYD-2/Liprin-α is a multifunctional scaffolding protein which is required for the transport of presynaptic proteins to the NMJ and has been shown to bind to motors and regulate their velocity and run length (Miller et al., 2005; Shin et al., 2003; Wagner et al., 2009). We investigated whether SYD-2 might also regulate the polarized trafficking of DCVs in DA/DB motor neurons.

Similar to our results in *cdk-5* mutants, we found a significant decrease in INS-22::Venus puncta fluorescence intensity in the motor neuron axons of two independent *syd-2* loss-of-function mutants, *syd-2(ju37)* and *syd-2(ok217)*(Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.04; *syd-2(ju37)*: 0.62±0.02; p<0.001; *syd-2(ok217)*: 0.74±0.02; p<0.001)(Fig. 2-3 A-E, G)(Ch'ng et al., 2008), and a significant increase in INS-22::Venus puncta intensity and density in the motor neuron dendrites (Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.02; *syd-2(ju37)*: 1.01±0.04, p>0.05; *syd-2(ok217)*:

CHAPTER 2
FIGURE S3

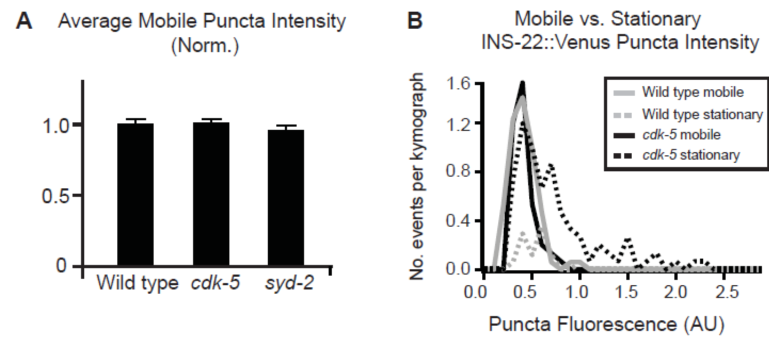
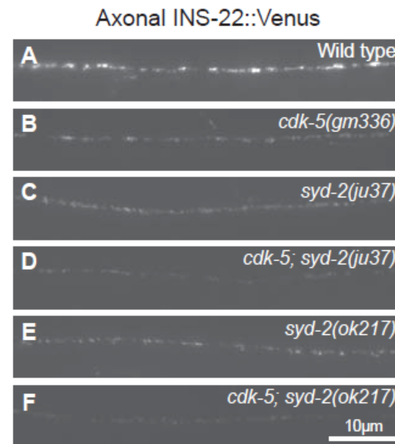
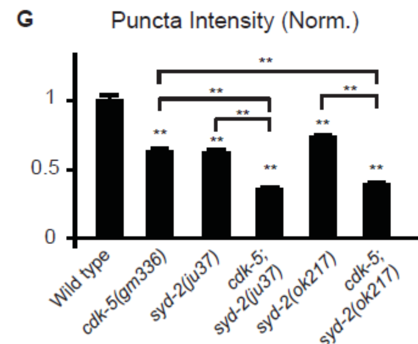
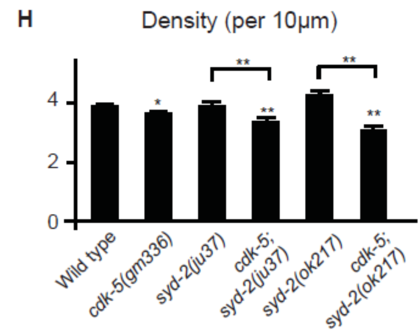


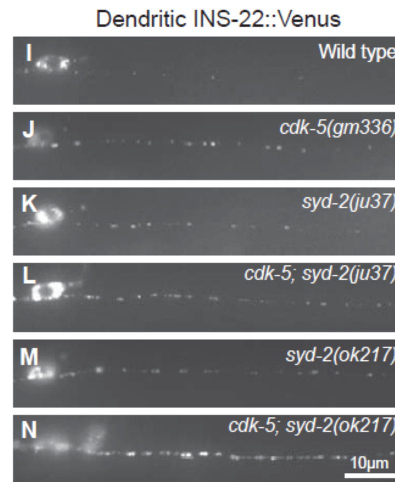
Figure 2-S3. Analysis of INS-22::Venus fluorescence in mobile versus stationary DCVs. (A) Average mobile INS-22::Venus puncta intensity, as calculated from kymographs, for wild-type (n=18), *cdk-5(gm336)*(n=21), and *syd-2(ju37)*(n=26) animals. No genotypes were significantly different from wild type (p>0.05, Student's *t* test). (B) Histogram analysis of INS-22::Venus puncta fluorescence intensities (AU) of mobile (solid grey line)(n=82) and stationary (n=17)(dotted grey line) puncta in wild type (grey) dendrites, and mobile (solid black line)(n=56) and stationary (dotted black line)(n=100) puncta for *cdk-5*(*gm336*) mutant dendrites.

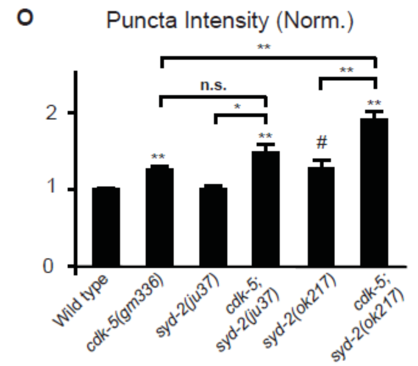
CHAPTER 2
FIGURE 3











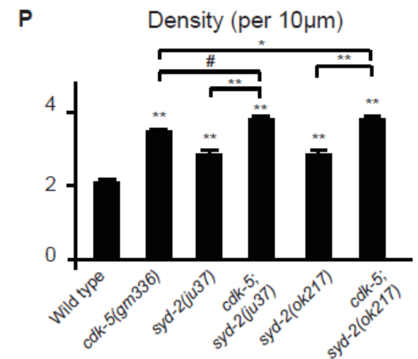


Figure 2-3. SYD-2/Liprin-α regulates the polarized distribution of DCVs in DA/DB motor neurons. (A-F) Representative images of INS-22::Venus in axons of young adult wild-type (A), *cdk-5(gm336)* (B), *syd-2(ju37)* (C), and *syd-2 (ok217)* (E) mutant animals. (D and F) Images of *cdk-5; syd-2(ju37)* (D) and *cdk-5; syd-2(ok217)* (F) double mutant animals. (G-H) Quantification of axonal INS-22::Venus puncta fluorescence intensity (G) and density (H) for wild-type (n=101), *cdk-5(gm336)* (n=46), *syd-2(ju37)*(n=25), *cdk-5;syd-2(ju37)* (n=23), *syd-2(ok217)*(n=24), and *cdk-5;syd-2(ok217)* (n=22) animals. (I-N) Representative images of INS-22::Venus in dendrites of young adult wild-type (I), *cdk-5(gm336)* (J), *syd-2(ju37)* (K), and *syd-2(ok217)* (M) mutant (H) animals. (L and N) Images of *cdk-5(gm336); syd-2(ju37)* (L) and *cdk-5(gm336); syd-2(ok217)* (N) double mutant animals. (O-P) Quantification of dendritic INS-22::Venus puncta fluorescence intensity (O) and density (P) for wild-type (n=138), *cdk-5(gm336)* (n=111), *syd-2(ju37)*(n=38), *cdk-5; syd-2(ju37) (n=24)*, *syd-2(ok217)*(n=28), *cdk-5(gm336); syd-2(ok217)* (n=28) animals. Values that differ significantly (Student's *t* test) are denoted #p<0.05, *p<0.01, **p<0.001 and n.s. p>0.05.

1.27±0.12; p<0.05) (Average puncta density (per 10 μm)±SEM: Wild type: 2.09±0.07; *syd-2(ju37)*: 2.84±0.13, p<0.001; *syd-2(ok217)*: 2.82±0.14; p<0.001)(Fig. 2-3, I-M, P). *syd-2* mutations did not affect DCV puncta density in motor neuron axons (Fig. 2-3H), or INS-22::Venus fluorescence in mobile puncta (p>0.05)(Fig. 2-S3). These data indicate that SYD-2/Liprin-α, like CDK-5, regulates the polarized distribution of DCVs in motor neurons.

CDK-5 and SYD-2 function in separate pathways

Our data show that *cdk-5* and *syd-2* single mutant animals have similar defects in the polarized distribution of DCVs in motor neurons suggesting that they may function in the same pathway. We tested this idea by analyzing the distribution of INS-22::Venus puncta in motor neuron axons of *cdk-5;syd-2* double mutant animals. As described above, INS-22::Venus puncta fluorescence decreases in *cdk-5*, *syd-2(ju37)* and *syd-2(ok217)* single mutant axons (Fig. 2-1 and 2-3). We found that INS-22::Venus puncta fluorescence also decreases in *cdk-5;syd-2(ju37)* and *cdk-5;syd-2(ok217)* double mutant axons and that this effect was greater than the decrease in the corresponding single mutants alone (Fig. 2-3 A-G)(Average puncta intensity(Norm.)±SEM: Wild type: 1.0±0.04; *cdk-5*: 0.63±0.03; *syd-2(ju37)*: 0.62±0.02; *cdk-5;syd-2(ju37)*: 0.36±0.02, p<0.001 vs *cdk-5*, p<0.001 vs *syd-2(ju37)*; *syd-2(ok217)*: 0.74±0.02, *cdk-5;syd-2(ok217)*: 0.39±0.02, p<0.001 vs *cdk-5*, p<0.001 vs *syd-2(ok217)*).

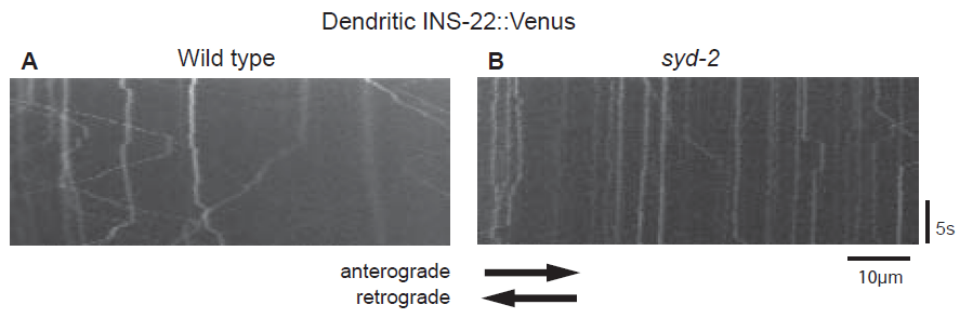
In motor neuron dendrites, the density of INS-22::Venus puncta increases in both *cdk-5* and *syd-2* single mutant animals (Fig. 2-1 and 2-3), and these effects are additive in *cdk-5;syd-2* double mutant animals compared to either single mutant (Fig. 2-3 I-O)(Average puncta density(per 10 μm)±SEM: Wild type: 2.09±0.07; *cdk-5*: 3.45±0.1; *syd-2(ju37)*: 2.84±0.13; *cdk-5;syd-2(ju37)*: 3.78±0.12, p<0.05 vs *cdk-5*, p<0.001 vs *syd-2(ju37)*; *syd-2(ok217)*: 2.82±0.14, *cdk-5;syd-2(ok217)*: 3.78±0.12, p<0.01 vs *cdk-5*, p<0.001 vs *syd-*

2(ok217)). Similarly, the intensity of INS-22::Venus puncta in *cdk-5*;*syd-2*(*ok217*) double mutants is greater than either single mutant (Fig. 2-3 I-O)(Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.02; *cdk-5*: 1.26±0.04; *syd-2(ok217)*: 1.27±0.12, *cdk-5;syd-2(ok217)*: 1.91±0.1, p<0.001 vs *cdk-5*, p<0.001 vs *syd-2(ok217)*). These results suggest that CDK-5 and SYD-2 function in separate pathways to regulate the polarized axonal-dendritic distribution of DCVs in motor neurons.

Because SYD-2/Liprin-α regulates the run length of SVs in *Drosophila* (Miller et al., 2005), and the velocity and run length of UNC-104/KIF1A in *C. elegans* (Wagner et al., 2009), we tested whether SYD-2 could regulate DCV motility. We performed time-lapse microscopy of mobile INS-22::Venus-containing DCVs in DA/DB motor neuron dendrites of wild-type and *syd-2* mutant animals (Fig. 2-4 and Table 2-1). Similar to our results in *cdk-5* mutants, we found a three-fold increase in the number of stationary DCVs in *syd-2* mutant dendrites relative to controls (Fig. 2-4 and Table 2-1). However, in contrast, we found a decrease in the amount of both anterograde and retrograde DCV trafficking (Fig. 2-4 and Table 2-1) in *syd-2* mutant dendrites. We also found that DCV run length is significantly decreased in both anterograde and retrograde directions in *syd-2* mutants compared to wild-type animals (Table 2-1). The different effects of *cdk-5* and *syd-2* mutation on DCV movement are consistent with the idea that CDK-5 and SYD-2 function in separate pathways to regulate the polarized distribution of DCVs.

Analysis of DCV movement in *cdk-5*;*syd-2* double mutants further supports this idea. We found that the amount of anterograde DCV trafficking in motor neuron dendrites of *cdk-5;syd-2* double mutants was significantly different from both *syd-2* (p<0.001) and *cdk-5* (p<0.001) single mutant animals (Fig. 2-S4 and Table 2-1). *cdk-5* single mutants have increased anterograde DCV trafficking in the dendrite relative to wild-type animals (Fig. 2-2). Similarly, anterograde DCV trafficking is increased in *cdk-5;syd-2* double mutants relative to

CHAPTER 2
FIGURE 4



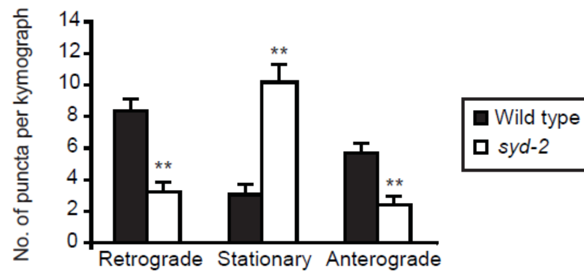


Figure 2-4. Effects of SYD-2/Liprin-α on DCV trafficking in motor neuron dendrites. (A-B) Representative kymographs generated from a 20 second movie of INS-22::Venus movement in young adult wild-type (n=18)(A) and *syd-2(ju37)*(n=26)(B) animals. (C) Quantification of the number of INS-22::Venus puncta observed moving anterogradely, retrogradely, or remaining stationary as calculated from kymographs of the two genotypes shown in (A-B).

CHAPTER 2

FIGURE S4

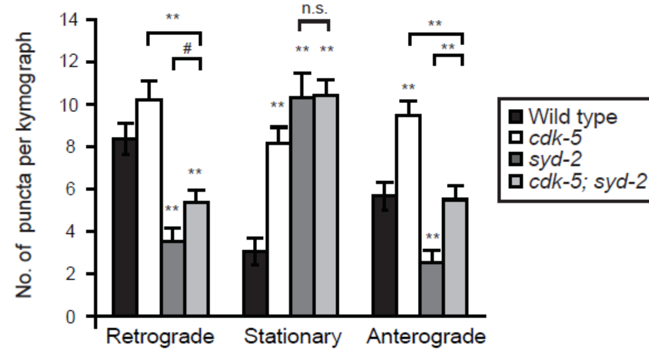


Figure 2-S4. CDK-5 and SYD-2/Liprin-α have additive effects on DCV trafficking in motor neuron dendrites. (A) Quantification of the number of INS-22::Venus puncta moving anterogradely, retrogradely, and remaining stationary during a 20 second kymograph in young adult wild-type (n=18), *cdk-5(gm336)*(n=23), *syd-2(ju37)*(n=26), and *cdk-5;syd-2* (n=22) double mutants. Values that differ significantly are denoted by asterisk (*) (#p<0.05, *p<0.01, **p<0.001, n.s p>0.05). See Table 1 for velocity and run length analysis.

syd-2 single mutant animals (Fig. 2-S4 and Table 2-1), indicating that *cdk-5* mutation can still increase the amount of dendrite-directed DCV trafficking even in the absence of *syd-2*. Overall, our analysis of *cdk-5* and *syd-2* single and double mutants suggests that CDK-5 and SYD-2 function in separate pathways to regulate the normal polarized axonal-dendritic distribution of DCVs.

The increased accumulation of DCVs in *cdk-5* mutant dendrites requires cytoplasmic dynein but not UNC-104/KIF1A motors

In order to better understand DCV trafficking in motor neurons, we used microtubule plus-end binding protein dynamics to determine the orientation of microtubules in both motor neuron axons and dendrites (Stepanova et al., 2003; Stone et al., 2008). In mammals, microtubules in the axon are oriented plus-end out from the cell body, whereas microtubules in the dendrite are of mixed polarity (Baas et al., 1988; Burton, 1988; Stepanova et al., 2003). In *Drosophila*, while microtubules in the axon are also oriented plus-end out, the majority of microtubules in dendrites are minus-end out (Stone et al., 2008). We made kymographs of EBP1-GFP, the *C. elegans* homolog of the plus-end microtubule binding protein EB1, to study microtubule polarity in both axons and dendrites of DA/DB motor neurons (see Materials and Methods). EBP-1::GFP puncta migrated at a velocity of 0.23±0.01 μm/sec, consistent with reports of EB1 motion in other systems (Mimori-Kiyosue et al., 2000; Morrison et al., 2002). In the axon, we found that microtubule orientation was predominantly (96%) plus-end out from the cell body (Fig. 2-S5), which is consistent with axonal microtubule orientation in both *Drosophila* and mammals (Baas et al., 1988; Stepanova et al., 2003; Stone et al., 2008). In the dendrite, we found that the orientation of microtubules was mixed, but that the majority of microtubules (84%) were oriented minus-

FIGURE S5

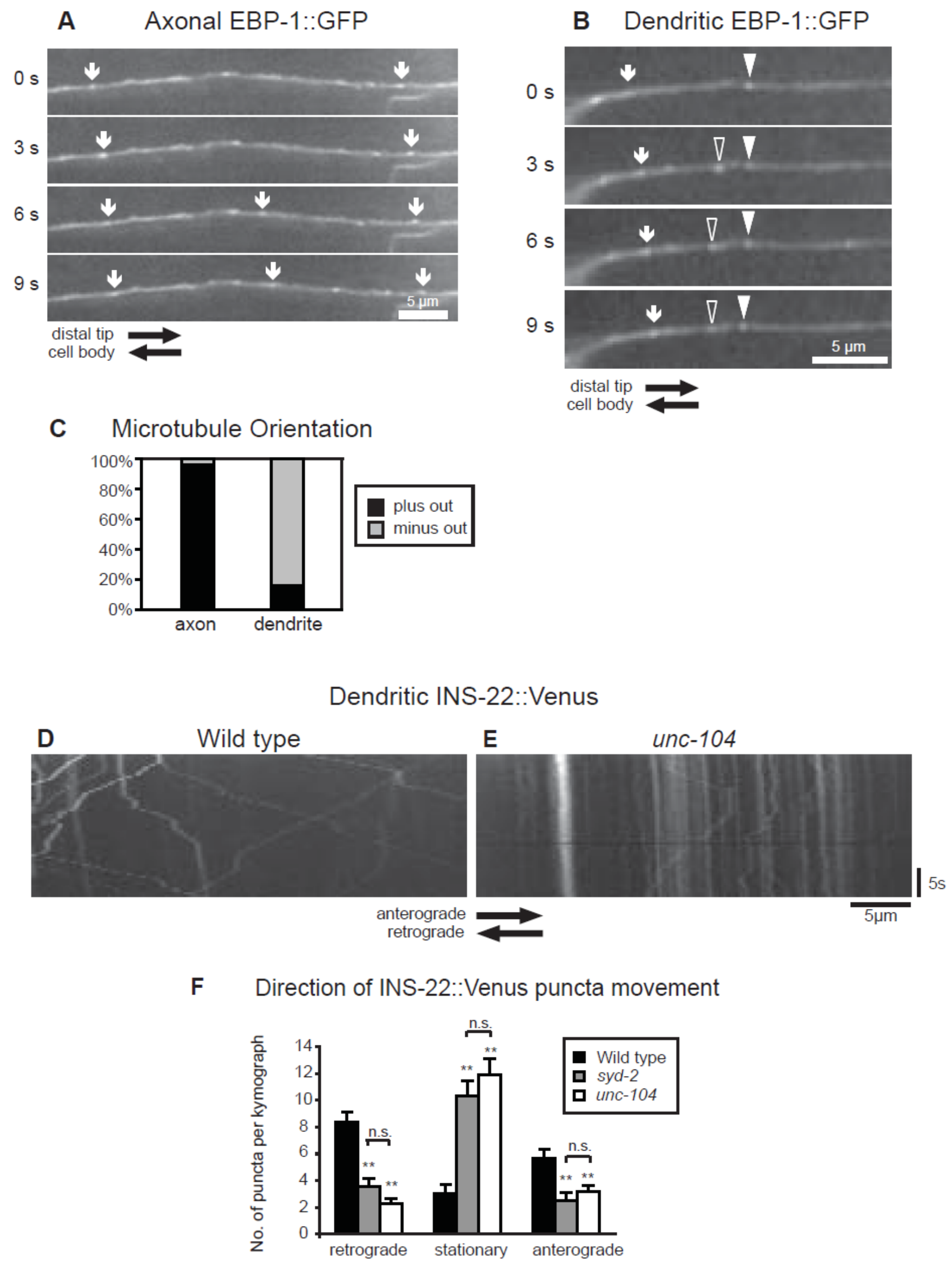


Figure 2-S5. Microtubule orientation in motor neuron axons and dendrites. (A-C) (A-B) Representative time-lapse frames of EBP-1::GFP movement in the axon (A) and dendrite (B). Arrows indicate plus-end out microtubule orientation. Arrowheads indicate minus-end out microtubule orientation. (C) Quantification of microtubule orientation in axons and dendrites of motor neurons based on EBP-1::GFP direction of movement (n = 72 puncta in axon, n = 64 puncta in dendrite). **SYD-2 and UNC-104 mutants have similar effects on DCV trafficking** (D-F) Representative kymographs generated from 20 second time-lapse movies of INS-22::Venus movement in wild-type (D) and *unc-104(e1265)* mutant (E) animals. (F) Quantification of the number of INS-22::Venus puncta observed moving anterogradely, retrogradely, or remaining stationary in kymographs. Values that differ significantly from wild type are denoted by asterisk (*) (**$p<0.001$, n.s. $p>0.05$).

end out from the cell body (Fig. 2-S5), which is similar to the dendritic microtubule orientation found in *Drosophila* motor neurons (Stone et al., 2008).

The kinesin UNC-104/KIF1A is a plus-end directed motor required for the transport of DCVs to axons in *C. elegans* and *Drosophila* (Barkus et al., 2008; Jacob and Kaplan, 2003; Pack-Chung et al., 2007; Sieburth et al., 2005; Zahn et al., 2004). Because our data indicate that DA/DB motor neuron dendrites contain some plus-end out microtubules, and UNC-104 has been reported to localize to dendrites as well as axons (Shin et al., 2003; Zhou et al., 2001), we tested whether the increase in dendritic DCVs observed in *cdk-5* mutants was dependent on UNC-104 by analyzing *cdk-5;unc-104* double mutant animals. Consistent with previous studies (Sieburth et al., 2005), we found a large decrease in the abundance of INS-22::Venus fluorescence in the motor neuron axons (data not shown) and a corresponding increase in the amount of INS-22::Venus in the cell bodies of *unc-104(e1265)* loss-of-function mutant animals (Average cell body intensity (Norm.)±SEM: Wild type: 1.0±0.07; *unc-104*: 1.81±0.12; $p<0.001$) (Fig. 2-5G-K). We also observed a significant increase in the abundance of INS-22::Venus fluorescence in the motor neuron dendrites of *unc-104* mutant animals compared to wild-type controls (Average puncta intensity (Norm.)±SEM: Wild type: 1.0±0.03; *unc-104*: 1.59±0.13; $p<0.001$)(Fig. 2-5A-F). This increase in INS-22::Venus puncta fluorescence in the motor neuron dendrites was further enhanced in *cdk-5;unc-104* double mutants compared to either *cdk-5* or *unc-104* single mutant animals (Average puncta intensity(Norm.)±SEM: Wild type: 1.0±0.07; *cdk-5(gm336)*: 1.3±0.05; *unc-104*: 1.81±0.12; *cdk-5;unc-104*: 2.25+0.13; $p<0.001$ vs *cdk-5*, $p<0.001$ vs *unc-104*)(Fig. 2-5 E). These results suggest that the increased accumulation of DCVs in the dendrites of *cdk-5* mutants does not require the kinesin UNC-104 and is thus not likely due to mistrafficking of UNC-104 into dendrites. The additive effects of *cdk-5* and *unc-104* on

CHAPTER 2

FIGURE 5

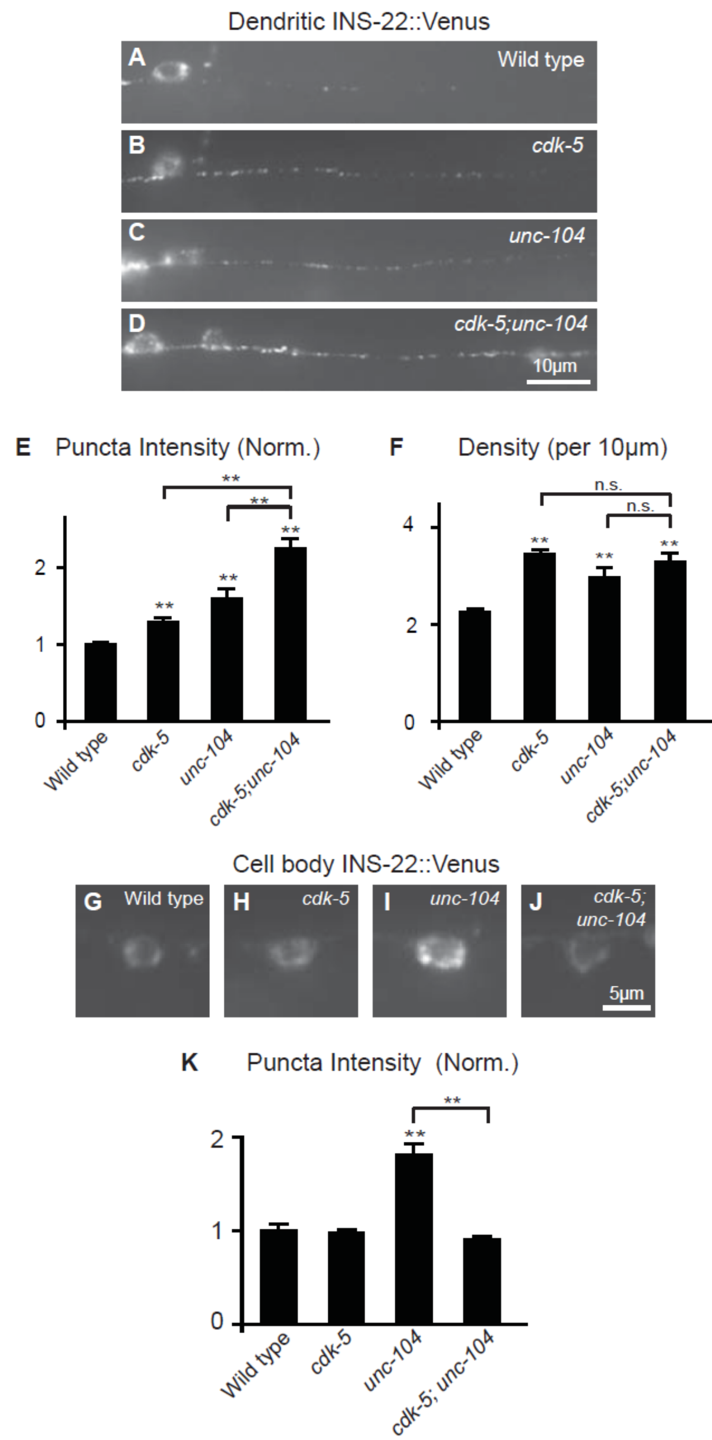


Figure 2-5. UNC-104/KIF1A is not required for DCV accumulation in *cdk-5* mutant dendrites. (A-D) Representative images of INS-22::Venus in dendrites of wild-type (A), *cdk-5(gm336)*(B), *unc-104(e1256)* (C), and *cdk-5;unc-104* double mutant (D) animals. (E-F) Quantification of dendritic INS-22::Venus puncta fluorescence intensity (E) and density (F) for wild-type (n=105), *cdk-5(gm336)*(n=103), *unc-104(e1265)*(n=32), and *cdk-5;unc-104* double mutants (n=25). (G-J) Representative images of INS-22::Venus in the motor neuron cell bodies of wild-type (G), *cdk-5(gm336)*(H), *unc-104(e1256)* (I), and *cdk-5;unc-104* double mutant (J) animals. (K) Quantification of somatic INS-22::Venus puncta fluorescence intensity for wild-type (n=25), *cdk-5(gm336)*(n=20), *unc-104(e1265)*(n=15), and *cdk-5;unc-104* double mutants (n=20)(** $p \leq 0.001$, n.s. $p > 0.05$).

dendritic DCV accumulation suggest that CDK-5 and UNC-104 function in separate pathways.

Interestingly, the accumulation of DCVs in the cell bodies of *unc-104* mutants decreased in *unc-104;cdk-5* double mutants concomitantly with the additive increase in the dendrites (Average cell body intensity (Norm.)±SEM: Wild type: 1.0±0.07; *cdk-5*: 0.97±0.04; *unc-104*: 1.81±0.12; *cdk-5;unc-104*: 0.9±0.04; p<0.001 vs *unc-104*)(Fig. 2-5 G-K). These results suggest that CDK-5 can function at the cell body to inhibit trafficking of DCVs into dendrites.

Kymograph analysis of mobile DCVs in the motor neuron dendrites of *unc-104* mutants showed an increase in stationary DCVs and a decreased number of both anterogradely and retrogradely moving DCVs (Avg. No. stationary puncta±SEM: Wild type: 3.1±0.6, *unc-104*: 11.9±1.2, p<0.01; Avg. No. anterograde puncta: Wild type: 5.7±0.7; *unc-104*: 3.2±0.5; p<0.01; Avg. No. retrograde puncta: Wild type: 8.4±0.7; *unc-104*: 2.3±0.4, p<0.01)(Fig. S5D-F). These changes in DCV movement in *unc-104* mutants were strikingly similar to those observed in *syd-2* mutants (Fig. 2-S5 F). Given that SYD-2 has been shown to cluster and regulate UNC-104 movement in *C. elegans* (Wagner et al., 2009), and *unc-104* and *syd-2* have similar defects in polarized DCV distribution and on the number and direction of DCV movement in dendrites, it is likely that SYD-2 and UNC-104 function together to promote axonal trafficking of DCVs. However, our genetic double mutant analysis of *cdk-5*;*syd-2* and *cdk-5*;*unc-104* animals suggest that CDK-5 functions in a separate pathway to both SYD-2 and UNC-104.

Because the majority of microtubules in the motor neuron dendrites are oriented minus-end out from the cell body, we tested whether the major minus-end directed motor, cytoplasmic dynein, was responsible for trafficking DCVs into *cdk-5* mutant dendrites. In addition, cytoplasmic dynein has been shown to traffic DCVs carrying BDNF and has

recently been shown to traffic pre- and postsynaptic cargoes into dendrites (Colin et al., 2008; Gauthier et al., 2004; Kapitein et al., 2010b; Ou et al., 2010). We found that mutations in the heavy chain of dynein, *dhc-1(js319)* (Koushika et al., 2004), completely block the increased accumulation of DCVs in dendrites observed in *cdk-5* mutants (Average puncta intensity(Norm.)±SEM: Wild type: 1.0±0.02; *cdk-5(gm336)*: 1.24±0.04; *dhc-1*: 0.96±0.06; *cdk-5;dhc-1*: 1.03±0.06; $p<0.001$ vs *cdk-5*) (Average puncta density(per 10 μm)±SEM: Wild type: 2.18±0.07; *cdk-5(gm336)*: 3.50±0.1; *dhc-1*: 2.06±0.35; *cdk-5;dhc-1*: 2.17±0.2; $p<0.001$ vs *cdk-5*) (Fig. 2-6). Taken together, these results suggest that cytoplasmic dynein is required for the accumulation of DCVs in the dendrites of *cdk-5* mutants and are consistent with a model where CDK-5 inhibits loading of DCVs onto dynein or trafficking of dynein-DCV complexes into dendrites.

CHAPTER 2
FIGURE 6

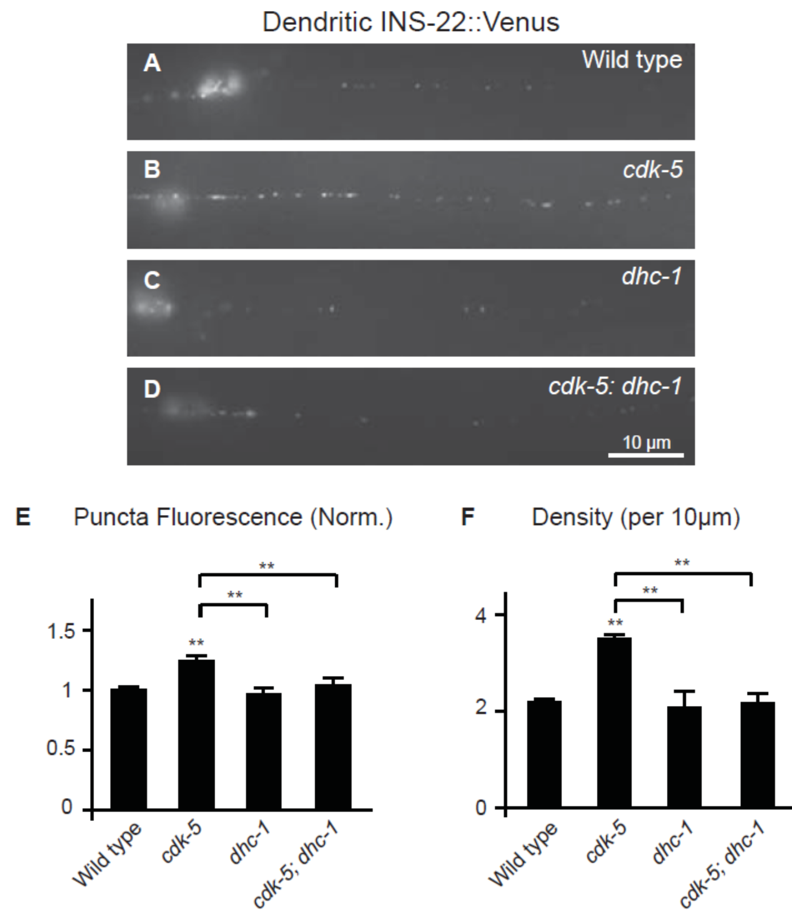


Figure 2-6. Mutations in cytoplasmic dynein block the increased accumulation of DCVs in *cdk-5* mutant dendrites. (A-D) Representative images of INS-22::Venus in dendrites of wild-type (A), *cdk-5(gm336)*(B), *dhc-1(js319)* (C), and *cdk-5;dhc-1* double mutant (D) animals. (E-F) Quantification of dendritic INS-22::Venus puncta fluorescence intensity (E) and density (F) for wild-type (n=139), *cdk-5(gm336)*(n=111), *dhc-1(js319)*(n=11), and *cdk-5;dhc-1* double mutants (n=21).

Discussion

Neurons are highly polarized cells and thus require high fidelity mechanisms to regulate the polarized trafficking of axonal and dendritic components. While much has been learned about the mechanisms involved in the polarized trafficking of axonal and dendritic transmembrane proteins (Arnold, 2009; Banker, 2003; Horton and Ehlers, 2003), less is known about the polarized trafficking of secreted proteins in neurons.

In this study, we show that CDK-5 and its activator CDKA-1/p35 are required for the normal polarized distribution of DCVs to axons of cholinergic DA/DB motor neurons. In *cdk-5* or *cdka-1* null mutant animals this polarized distribution is disrupted and INS-22::Venus-containing DCVs accumulate in motor neuron dendrites (Fig. 2-1). Analysis of mobile DCVs in *cdk-5* mutants revealed an increase in the number of DCVs trafficking into dendrites and accumulating in dendrites (Fig. 2-2 and Table 2-1). Since we found that the majority of microtubules in these dendrites are oriented with their minus-end out from the cell body (Fig. 2-S5), we tested whether the minus-end directed motor cytoplasmic dynein was involved in this process. Consistent with this idea, we found that mutations in cytoplasmic dynein heavy chain, *dhc-1*, completely block the increased accumulation of DCVs in dendrites of *cdk-5* mutants (Fig. 2-6). Mutations in the scaffolding protein SYD-2/Liprin-α and the axonal DCV motor UNC-104/KIF1A also result in defects in the polarized distribution of DCVs (Fig. 2-3 and 2-5). However, genetic analysis of *cdk-5*, *syd-2* and *unc-104* single and double mutants suggest that SYD-2 and UNC-104 likely function together to promote axonal trafficking of DCVs, whereas CDK-5 functions in a separate pathway to both SYD-2 and UNC-104 to regulate polarized DCV trafficking (Fig. 2-3, 4, 5, S4, S5 and Table 2-1).

There are several potential models to explain the mechanism by which CDK-5 regulates polarized trafficking of DCVs: (1) The axonal DCV motor UNC-104 mistraffics into dendrites in *cdk-5* mutants. We think this model is unlikely. In *unc-104* mutants, DCVs do

not traffic properly to the axon and accumulate in the motor neuron cell bodies and dendrites (Fig. 2-5)(Sieburth et al., 2005). If this model is correct, then genetically removing *unc-104* in a *cdk-5* mutant background should not increase the DCV trafficking defect of *cdk-5* single mutants (i.e. the mutations should be non-additive). Instead, we found that *cdk-5;unc-104* double mutants had increased abundance of DCVs in the dendrites compared to either single mutant alone (Fig. 2-5). (2) CDK-5 positively regulates axonal trafficking of DCVs on UNC-104. For example, CDK-5 might directly promote the loading of DCVs onto the axonal kinesin UNC-104 or promote anterograde trafficking of UNC-104 into axons. If this model is correct, then *cdk-5;unc-104* double mutants should have a non-additive effect on DCVs in the dendrites. We think this model is unlikely because, as described above, *cdk-5;unc-104* double mutants have additive increases in DCVs in dendrites (Fig. 2-5). (3) DCVs are trafficked to both axons and dendrites and CDK-5 promotes retrograde trafficking of DCVs from dendrites back to the cell bodies. If this model is correct, *cdk-5* mutants should have a decrease in the number of retrogradely moving DCVs. In contrast, our time-lapse analysis of mobile DCVs showed that *cdk-5* mutants have increased numbers of anterogradely moving DCVs in dendrites with no significant change in the number of retrogradely moving DCVs (Fig. 2-2), suggesting that this model is unlikely. (4) CDK-5 inhibits the transport of DCVs into dendrites. Our data are most consistent with this model. First, *cdk-5* mutants have increased numbers of anterogradely moving DCVs in dendrites (Fig. 2-2). Second, the majority of microtubules in these dendrites are oriented minus-end out from the cell body (Fig. 2-S5) and mutations in the minus-end directed motor cytoplasmic dynein completely block the increased accumulation of DCVs in *cdk-5* mutant dendrites (Fig. 2-6). Third, the cell body accumulation of DCVs observed in *unc-104* single mutants is reduced to wild type levels in *cdk-5;unc-104* double mutants along with a concomitant increase of DCVs in the dendrites (Fig. 2-5). Thus, we propose that CDK-5 functions at the cell body to either inhibit

DCV loading onto a dendrite-directed motor (i.e. dynein) or prevent this motor from trafficking into dendrites.

This study also identifies SYD-2 as a novel regulator of DCV polarity. We show that mutations in the scaffolding protein SYD-2 or the axonal DCV motor UNC-104 also affect the normal polarized distribution of DCVs in motor neurons (Fig. 2-3 and 2-5). Although *syd-2*, *unc-104* and *cdk-5* single mutants have similar defects in the polarized distribution of DCVs, our genetic analysis of *cdk-5;syd-2* and *cdk-5;unc-104* double mutants indicate that CDK-5 functions in a separate pathway to both UNC-104 and SYD-2. SYD-2/Liprin-α is a multifunctional protein that plays a central role in presynaptic active zone assembly and development in *C. elegans* and *Drosophila* (Dai et al., 2006; Kaufmann et al., 2002; Patel et al., 2006; Zhen and Jin, 1999), and in regulating the trafficking of synaptic proteins. In mammalian neurons, SYD-2 regulates the trafficking of postsynaptic proteins (Dunah et al., 2005; Ko et al., 2003; Wyszynski et al., 2002), and in *Drosophila*, SYD-2 can associate with kinesin-1 motors and positively regulate SV run length (Miller et al., 2005). In *C. elegans*, *Drosophila* and mammals, UNC-104/KIF1A is required for transporting SVs and DCVs to axons (Barkus et al., 2008; Hall and Hedgecock, 1991; Jacob and Kaplan, 2003; Okada et al., 1995; Pack-Chung et al., 2007; Sieburth et al., 2005; Zahn et al., 2004). Because SYD-2/Liprin-α can bind and cluster UNC-104/KIF1A *in vitro* and regulate its velocity and run length in *C. elegans* neurons (Shin et al., 2003; Wagner et al., 2009), and our data show that *syd-2* and *unc-104* mutations have similar effects on the number and direction of DCV movement in dendrites (Fig. 2-S5), it seems likely that SYD-2 functions together with UNC-104 to promote axonal DCV trafficking. Interestingly, a recent study in *Drosophila* showed that mutations in *unc-104* can affect bidirectional DCV movements suggesting that UNC-104 may also regulate retrograde DCV trafficking (Barkus et al., 2008). Consistent with this notion, our kymograph data also show that *syd-2* and *unc-104* affect bidirectional DCV

movements in *C. elegans* (Figs. 2-4 and 2-S5). However, some aspects of DCV mobility differ between *unc-104* and *syd-2* mutants (Table 2-1), suggesting that these genes may also have some non-overlapping functions. Further experiments will be necessary to determine the exact mechanisms by which SYD-2 and UNC-104 regulate DCV movement in dendrites.

In summary, we propose the following model to explain the role of CDK-5 in regulating polarized DCV trafficking in DA/DB motor neurons (Fig. 2-7). Mutations in *cdk-5*, *syd-2* or *unc-104* result in defects in the normal polarized axonal-dendritic distribution of DCVs in motor neurons. SYD-2 likely functions together with the kinesin UNC-104 to promote trafficking of DCVs from the cell body into axons. CDK-5 functions in a separate pathway from SYD-2 and UNC-104 (i.e. in *syd-2* or *unc-104* mutants, DCVs accumulate in the dendrite via a mechanism that functions in parallel to CDK-5). CDK-5 prevents the trafficking of DCVs into dendrites either by inhibiting the loading of DCVs onto a dendrite-directed motor (i.e. the microtubule minus-end directed motor dynein) or by inhibiting trafficking of this motor into dendrites. Thus, the normal polarized distribution of INS-22::Venus-containing DCVs to axons requires not only axonal transport by UNC-104, but also active inhibition of dendritic trafficking by CDK-5.

SV precursors are also trafficked in a polarized manner to axons and several genes and mechanisms have been described. In cultured mammalian hippocampal neurons, sequences in the cytoplasmic domain of Synaptobrevin are involved in trafficking SVs to axons (West et al., 1997). In *C. elegans* motor neurons, the JNK scaffolding protein UNC-16/JIP3, the motor UNC-116/Kinesin-1, and the kinase LRK-1 regulate the polarized trafficking of SVs to axons (Byrd et al., 2001; Sakaguchi-Nakashima et al., 2007; Sakamoto et al., 2005), and an extracellular Netrin signal can inhibit the inappropriate localization of

CHAPTER 2

FIGURE 7

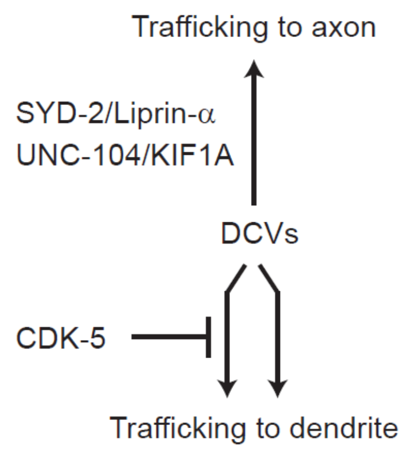


Figure 2-7. Model for CDK-5 and SYD-2/Liprin-α regulation of DCV trafficking in motor neuron dendrites. SYD-2 likely functions with the microtubule plus-end directed motor UNC-104 to promote trafficking of DCVs from the cell body into axons. CDK-5 prevents the trafficking of DCVs in the cell body into dendrites by either preventing DCV loading onto a dendrite-directed motor (i.e. dynein) or by inhibiting trafficking of this motor into dendrites. CDK-5 inhibition of dendrite-directed trafficking of DCVs likely occurs in the cell body as well as the axon.

SVs to dendrites (Poon et al., 2008). Interestingly, a recent study showed that CDK-5 and another cyclin-dependent kinase PCT-1 function in parallel to regulate the polarized trafficking of SV precursors to axons and also proposed that these kinases function by inhibiting dynein-dependent trafficking of SVs into dendrites (Ou et al., 2010). It will be interesting to test if PCT-1 also functions in parallel with CDK-5 to regulate polarized DCV trafficking. Taken together with our data, these studies suggest that SV precursors and INS-22-containing DCVs destined for the axon appear to share the same CDK-5-dependent mechanisms for polarized trafficking. Because DCVs differ from SVs in that they transport diverse cargos, including cargo destined for secretion in dendrites, much remains to be learned about the molecular mechanisms involved in trafficking and targeting of different DCV populations to specific destinations.

Materials and Methods

Strains

Strains were maintained at 20°C as described by Brenner et al (1974). OP50 *E. coli* strain was used for feeding for all strains. The following strains were used in this study: N2 Bristol*, nuIs195 (Punc-129::Ins-22::Venus), ceIs72(Punc-129::IDA-1::GFP), nuIs168 (Punc-129::Rab-3::Venus), nuIs165 (Punc-129::unc-10::GFP), nuIs174 (Punc-129::Rab-3::GFP), pzEx77 (Punc-17::cdk-5), pzEx107(Punc-17::cdk-5(D144N)), pzEx114(Punc-129::cdka-1), pzEx140(Punc129::cdk-5), pzEx133(Punc129::fibrillin::GFP), pzEx156(Punc129::EBP-1::GFP), yuEx46(Punc129::UNC-9::GFP), cdk-5(gm336), cdk-5(ok626), cdka-1(gm335), syd-2(ju37), syd-2(ok217), unc-10(e102), unc-104(e1265), dhc-1(js319).* The *cdk-5* allele *gm336* is a predicted null mutation and consists of a 760 bp deletion that eliminates the start codon as previously described (Juo et al., 2007). The *ok626* allele is also a predicted null mutation that consists of a 1.6kb deletion that eliminates the *cdk-5* start codon but also deletes part of a neighboring gene T27E9.4. The *syd-2* mutant alleles have been previously described (Wagner et al., 2009; Zhen and Jin, 1999).

Constructs, transgenes, and germline transformation

Plasmids were generated using standard cloning techniques and details are available upon request. *Punc-129::cdk-5* (FJ#55) and *Punc-129::cdka-1* (FJ#56) were generated by subcloning *cdk-5* from *pV6::cdk-5* (KP#1414) and *cdka-1* from *pV6::cdka-1* (KP#1413) into the *Punc-129* expression vector KP#1271. Wild-type and kinase-dead *cdk-5* were subcloned from *pV6::cdk-5* (KP#1414) and *pV6::cdk-5(D144N)* (KP#1415), respectively, under the control of the *unc17* promoter. The *fibrillin* and *ebp-1* open reading frame were obtained by RT-PCR from wild-type cDNA and subcloned under the control of the *unc-129* promoter. NotI sites were introduced at the C-termini of both genes and used to insert GFP

to create *Punc-129::fibrillin::GFP* (FJ#57) and *Punc-129::ebp-1::GFP* (FJ#62). Transgenic strains were generated by microinjection of various plasmids at the following concentrations: 50 ng/μl for *Punc-17::cdk-5(wt), Punc-17::cdk-5(D144N), Punc-129::cdk-5, Punc-129::cdka-1*, 25ng/μl for *Punc-129::fibrillin::GFP,* and 1ng/μL for *Punc129::EBP-1::GFP*. Plasmids were injected with various co-injection markers: *Pttx-3::dsRED*, *Pmyo-2::NLS::GFP*, or *Pmyo-2::NLS::cherry.*

Fluorescent Microscopy and Quantification

All imaging was performed using a Zeiss M1 Axioimager microscope. For all experiments, except for time-lapse imaging, young adult animals were immobilized with 30mg/mL 2,3-Butanedione monoxamine (Sigma Aldrich) for 5-7 minutes and mounted on 2% agarose pads prior to imaging. Images were taken using a Zeiss 100X planapo objective (NA 1.4) and an Orca-ER (Hamamatsu) CCD camera. Maximum intensity projections of Z-series stacks and line scans of fluorescent puncta were obtained using Metamorph (v7.1) software (Molecular Devices). For quantitative analyses of fluorescent ventral and dorsal nerve cord puncta, maximum intensity projections of Z series stacks (total depth 1μm) were made. Exposure settings and gain were set to fill the 12-bit dynamic range without saturation and were constant for all images of a given fluorescent marker. FluoSphere yellow-green fluorescent beads (Invitrogen) were imaged daily to correct for day-to-day variation in microscope light bulb intensity. All images were taken from animals oriented with the dorsal or ventral nerve cord up and laterally oriented animals were excluded. For all images except time-lapse, images were taken posterior to the vulva. Images containing mobile INS-22::Venus puncta were excluded from analysis. For dorsal (axons) and ventral (dendrites) nerve cord images, line scans were drawn along DA6 or DB6 processes. Line scans of nerve cord puncta were generated using MetaMorph (v6.0) and were then analyzed in Igor

Pro (v5) using custom written software as previously described (Burbea et al., 2002). Puncta intensity and density were calculated from each image. Puncta intensity is the fractional increase in peak fluorescence of each puncta over fluorescent bead intensity for that date. Puncta density is the average number of puncta per 10 μm of nerve cord. For DA6/DB6 cell body imaging, lateral and ventral up animals were imaged, and Z stacks were taken to a depth of 2 μm. Average fluorescence of three fluorescent patches per cell body was measured using Metamorph software, then corrected for daily bead values and analyzed in Microsoft Excel.

Time-lapse microscopy

All time-lapse imaging was performed using a Zeiss M1 Axioimager microscope using the 100x objective. For time-lapse microscopy, young adult worms were paralyzed in 360μg/mL Levamisol (Sigma) dissolved in M9 buffer for 6 to 7 minutes. Animals were mounted on 2% agarose pads containing 360 μg/mL Levamisol. Images were taken of a region of the VNC between DA2 and DA3 motor neuron cell bodies from animals oriented with their VNC facing the objective. Because DA motor neuron dendrites project anteriorly, all INS-22::Venus puncta traveling towards the head in this region were designated as anterogradely moving, and all puncta traveling towards the tail were designated as retrogradely moving. Time-lapse images were taken at 4 Hz speed for 20 seconds and saved as a Z series. Line scans of the ventral nerve cord were taken between DA3 and DA2 motor neuron cell bodies, with all scans beginning at the DA3 cell body. These scans were used to generate kymographs in MetaMorph (v7.1). INS-22::Venus puncta direction of movement, run length, and velocity were calculated by tracing these kymographs. For direction of movement and velocity analyses, each mobile puncta was traced during its longest uninterrupted period of movement. Puncta were defined as mobile if they moved

distances greater than twice their own width and at velocities greater than 0.1 μm/s. Puncta that changed direction were traced for both directions. For run length analysis, puncta were traced for all qualifying movements, not just the longest uninterrupted period of movement, and were only included if they began and finished a movement during the movie. Kymograph traces were compiled for analysis in Microsoft Excel. Average anterograde and retrograde velocity and run length, and the number of stationary, anterogradely, and retrogradely moving puncta were calculated for each kymograph (i.e. worm) and these data were compiled for each genotype and analyzed in Microsoft Excel. Statistical analysis of average velocity and direction of movement was performed by using *F* tests to determine if data sets had equal variances at a 95% confidence interval, followed by Student's *t* tests to detect differences between genotypes. The Kolmogorov-Smirnov (KS) test was used to analyze significant differences in run length.

Kymograph analysis using Metamorph software was also used to measure the fluorescence intensity of mobile INS-22::Venus puncta in dendrites. The maximum fluorescence intensity of each mobile punctum was measured at the t=0 time point of each kymograph, to minimize the effects of photobleaching. Maximum intensity measurements were analyzed using Microsoft Excel and IgorPro (v5). Occasionally, mobile INS-22::Venus puncta were observed to undergo a splitting event, where a single punctum divides into two separate puncta during the time-lapse movie. While these events are rare, it suggests that a mobile punctum may represent more than one DCV. For this reason, splitting puncta were excluded from mobile INS-22::Venus fluorescence intensity analyses in order to enrich for puncta representing single DCVs.

For time-lapse microscopy of EBP-1 movement, animals were paralyzed in 2mM Levamisol dissolved in M9 for 8 to 9 minutes and mounted on a 2% agarose pad containing 2mM Levamisol. Images were taken of dorsal and ventral nerve cords in regions either

anterior to the DA3 cell body or posterior to the DB6 cell body, where EBP-1::GFP (under control of *Punc-129*) is expressed in DA and DB processes, respectively. Our analysis of EBP-1::GFP in DA3 dendrites may underestimate the proportion of minus-end out microtubules because the posterior process of DB3 may express a low level of EBP-1::GFP. Time-lapse images were taken at a speed of 4Hz for 25s. Line scans were taken from the cell body outwards and used to generate kymographs. EBP-1::GFP direction of movement and velocity were calculated by tracing puncta in these kymographs. Only puncta that could be followed for 2s or more were included. Occasionally, oscillating puncta were visible (consistent with reports from (Stepanova et al., 2003)), and these puncta were excluded from analysis. Direction of movement and velocity of EBP-1::GFP puncta were analyzed using Microsoft Excel.

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CHAPTER 3

Mechanisms of cyclin-dependent kinase-5 regulation of neuropeptide abundance in *C. elegans* motor neuron axons

Attributes: All experiments in the following chapter were performed by Patricia Goodwin.

Introduction

Fast microtubule-based transport of presynaptic cargoes down the axon is critical for synaptic function and neuronal survival. While some recent studies show that local translation of proteins can occur in axon growth cones (Lin and Holt, 2008), axon terminals rely on fast axonal transport for delivery of organelles and proteins from the cell body to maintain synaptic function (De Vos et al., 2008). Loss of axonal transport can lead to axon retraction, dying-back neuropathies, and neurodegeneration (De Vos et al., 2008).

A variety of neurodegenerative diseases and motor neuron diseases can be caused by motor mutations and alterations in fast axonal transport (De Vos et al., 2008; Reid et al., 2002). Mutations in Kif1Bβ have been linked to Charcot-Marie-Tooth disease (Zhao, 2001). Kif5/kinesin-1 mutations have been linked to a rare form of hereditary spastic paraplegia (Reid et al., 2002). Similarly, mutations in members of the dynactin complex have been linked to both amyotrophic lateral sclerosis (ALS) and a L-DOPA resistant form of Parkinson's Disease called Perry's disease (Eschbach and Dupuis, 2011). Disrupted anterograde and retrograde trafficking has been observed early in the pathogenesis of motor neuron diseases including ALS and spinal and bulbar muscular atrophy (Eschbach and Dupuis, 2011). Furthermore, many of the neurodegenerative diseases that are characterized by intracellular protein aggregates, including ALS, Huntington's Disease (HD), and Alzheimer's Disease (AD), also show signs of disrupted axonal transport as an early pathological event in animal models (De Vos et al., 2008).

Cyclin-dependent kinase-5 is a serine/threonine kinase that has been implicated both in neurodegeneration and in the regulation of axonal transport (Dhavan and Tsai, 2001; Morfini et al., 2004; Tsai et al., 2004). Many of CDK-5's substrates are important for microtubule-based fast axonal transport (Dhavan and Tsai, 2001). CDK-5 inhibitors decrease anterograde trafficking of membrane-bound organelles in the axon by

antagonizing the activity of GSK-3, which leads to the disassociation of Kif5/kinesin-1 from its cargoes (Morfini et al., 2004). This regulation may be relevant to the pathology of AD, as mutations in Presenilin-1 that are linked to familial AD cause defects in Kif5-mediated axonal transport through up-regulation of GSK-3 activity (Lazarov et al., 2007). Recently, CDK-5 and another cyclin-dependent kinase, PCT-1/Pctaire kinase, have been shown to promote presynaptic localization of synaptic vesicles (SVs) by inhibiting dynein-mediated retrograde trafficking (Ou et al., 2010). In addition, CDK-5 may regulate motor movement by phosphorylating microtubule associated proteins (MAPs), which can bind to microtubules to regulate their polymerization, stability, and interactions with motors (Dehmelt and Halpain, 2005; Dixit et al., 2008). Several MAPs, including tau, MAP1B, doublecortin, and CRMP2, are CDK-5 substrates (Dhariwala and Rajadhyaksha, 2008; Dhavan and Tsai, 2001; Tanaka et al., 2004; Tsai et al., 2004). When activated by p25, CDK-5 can hyperphosphorylate tau and induce neurofibrillary tangle formation, a hallmark of AD (Fischer et al., 2005; Lee et al., 2000; Patrick et al., 1999). Expression of p25 can increase mitochondrial pausing during axonal transport (Morel et al., 2010) and p35 itself has also been shown to bind microtubules and regulate their dynamics (Hou et al., 2007). Thus, by regulating either motors themselves or the microtubules they travel on, CDK-5 activity can regulate axonal transport.

The transportation of dense-core vesicles (DCVs) may be particularly relevant to neurodegenerative disorders, because trophic factors including BDNF are transported in DCVs. Impaired transport of brain-derived neurotrophic factor (BDNF)-containing DCVs has been suggested to be crucial to the development of HD, because wild type *huntingtin* expression promotes BDNF transport, while mutant *huntingtin* expression impairs BDNF transport (Caviston and Holzbaur, 2009). Overexpression of BDNF can restore motor function in mouse models of HD, suggesting loss of BDNF is a key part of disease

pathogenesis (Xie et al., 2010). In the previous chapter, we identified CDK-5 as a novel regulator of DCV transport. We showed that *C.elegans cdk-5* mutants have increased dendritic DCVs due to loss of inhibition of dynein and a concomitant decrease in axonal DCVs. The simplest explanation for these two changes arising from a single mutation is that the change in the dendrite causes the change in the axon (or vice versa). However, a recent study found that CDK-5 also inhibits the dendritic trafficking of SVs, but appears to not affect axonal localization of SVs (Ou et al., 2010). The authors find instead that loss of *cdk-5* can be compensated for in the axon by PCT-1/Pctaire kinase. Interestingly, these two kinases perform redundant roles to promote axonal trafficking in DA cholinergic motor neurons, but surprisingly are dispensable in DB cholinergic neurons, suggesting CDK-5 has cell-type specific effects. These cell-type specific results are intriguing but unexpected because DA and DB motor neurons have similar morphologies and functions. Seemingly, these neurons only differ in that DAs have anteriorly-projecting processes and are involved in backwards locomotion, while DBs have posteriorly-projecting processes and are involved in forward locomotion. These findings raised some interesting questions regarding CDK-5's role in axonal trafficking of vesicles: (1) Does CDK-5 regulate DCVs and SVs through similar mechanisms? (2) Does it regulate the trafficking of DCVs in a cell-type specific manner? (3) Is CDK-5's inhibition of dendritic trafficking linked to its role in promoting axonal transport?

In this chapter, we further characterize the role that CDK-5 plays in promoting DCV localization to the axon and also examine a potential role for CDK-5 in regulation of DCV release. Decreased INS-22::Venus in axons of *cdk-5* mutants (Chapter 2, Fig 2-1) could be attributed to increased release of neuropeptides. CDK-5 has been shown to negatively regulate synaptic vesicle release in hippocampal and striatal neurons (Chergui et al., 2004; Kim and Ryan, 2010). CDK-5 activity also negatively regulates release of vesicles from chromaffin cells (Barclay et al., 2004), PC12 cells (Amin et al., 2008), and pancreatic beta

cells (Wei et al., 2005). Given that CDK-5 inhibits SV and DCV release in multiple cell types, *cdk-5* mutants may have fewer neuropeptide markers in the axon due to increased neuropeptide release.

In this chapter, we also explore the role of two cyclin-dependent kinase signaling pathways, CDK-5 and PCT-1/CYY-1, in regulation of axonal localization of neuropeptides in DA and DB motor neurons. We demonstrate that while CDK-5 and CYY-1 play redundant roles in the regulation of axonal trafficking of DCVs in DA motor neurons, CDK-5 has a unique role in axonal trafficking in DB neurons that cannot be compensated for by PCT-1/CYY-1. We also show that CDK-5 regulates neuropeptide abundance in the axon through two different mechanisms: regulation of anterograde trafficking of DCVs and regulation of neuropeptide release.

Results

CDK-5 is required for axonal trafficking of DCVs in DB motor neurons but not DA

Given that Ou et al (2010) observed differences in CDK-5's regulation of SV trafficking between DA and DB motor neurons, we wondered whether the decrease in axonal DCVs we observed in the previous chapter generalizes to all cholinergic motor neurons, or if it is a cell-type specific effect. The *Punc129* promoter we used to express INS-22::Venus drives expression in nine DA and DB motor neurons (Fig. 3-1). Ou et al (2010) were able to separate DA and DB through electromicroscopic reconstruction and by the use of a DA specific promoter. Although no DB-specific promoters have been published, we can enrich our analysis of proteins in DB neurons versus DA neurons by using the *unc129* promoter and imaging proteins in the posterior versus anterior. The *unc-129* promoter drives expression in more DAs in the anterior and more DBs in the posterior (Fig.3 -1, see Materials and Methods). Our data in Chapter 2 are derived from a mix of INS-22::Venus

CHAPTER 3
FIGURE 1

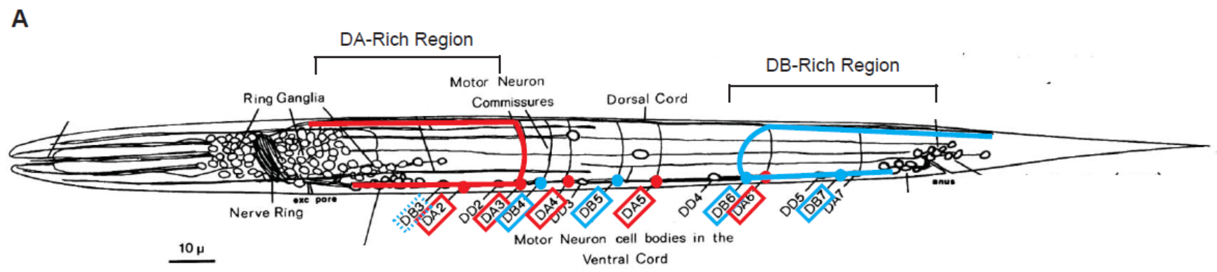

Figure 3-1. Diagram of *Punc-129*-driven expression in DA and DB neurons (A) A truncated form of the *unc-129* promoter drives expression of genes in 9 to 10 DA and DB neurons. DA2, DA3, DB4, DA5, DB5, DA5, DB6, DA6, and DB7 show fully penetrant, robust expression of *Punc-129::INS-22::Venus* in the neuronal cell body. Weak expression of *Punc129::INS-22::Venus* is found in DB2 neuronal cell bodies in less than 25% of wild type animals. DA neurons have anteriorly projecting dendrites and axons, while DB neurons have posteriorly projecting dendrites and axons. For clarity, neuronal processes are only drawn for one DA neuron (DA3) and one DB neuron (DB6). The length of neuronal processes are not to scale. Adapted from White and Durbin, 1988.

signal from dendrites of both DA and DB neurons, however because we imaged axonal DCVs in the posterior in our initial studies, our axonal data are derived mostly from DB neurons. To contrast DB axons to DA axons, we repeated the imaging of INS-22::Venus in the anterior of the animal where most of the neurons expressing INS-22::Venus are DA neurons.

Surprisingly, when axonal INS-22::Venus fluorescence was analyzed in the DA axon-rich region rather than the DB-rich region, there was no longer any difference between *cdk-5* mutants and wild type animals. Axonal INS-22::Venus puncta fluorescence intensity and density were not significantly altered in *cdk-5* mutants relative to wild-type controls (Fig. 3-2 A-F). In contrast, *cdk-5* mutants had significantly reduced INS-22::Venus puncta fluorescence ($p<0.001$) and puncta density ($p<0.01$) in the DB axon compared to wild type animals. This demonstrates that CDK-5's effect on DCV localization to the axon is specific for DB neurons.

Because we found a DB-specific role for CDK-5 in regulation of DCV localization in the axon, we wondered if there are also cell-type specific differences in dendritic trafficking of DCVs in *cdk-5* mutants. In contrast to our findings in the axon, both DA and DB neurons had increased DCV localization in the dendrites of *cdk-5* mutants relative to wild type animals (Fig. 3-2 G-L). In the case of DA neurons, *cdk-5* mutants had significantly increased puncta fluorescence and density compared to wild-type animals (Fig. 3-2 K-L). In DB neurons, INS-22::Venus puncta density was also significantly increased in *cdk-5* mutants (Fig.3- 2 K-L). These data show that CDK-5 negatively regulates the abundance of DCVs in dendrites of DCVs in both DA and DB neurons, but only regulates axonal abundance of DCVs in DB neurons. These results uncouple changes in DCV abundance in axons and dendrites and suggest that loss of DCVs from the axon may not be caused by increased dendritic trafficking of DCVs.

CHAPTER 3
FIGURE 2

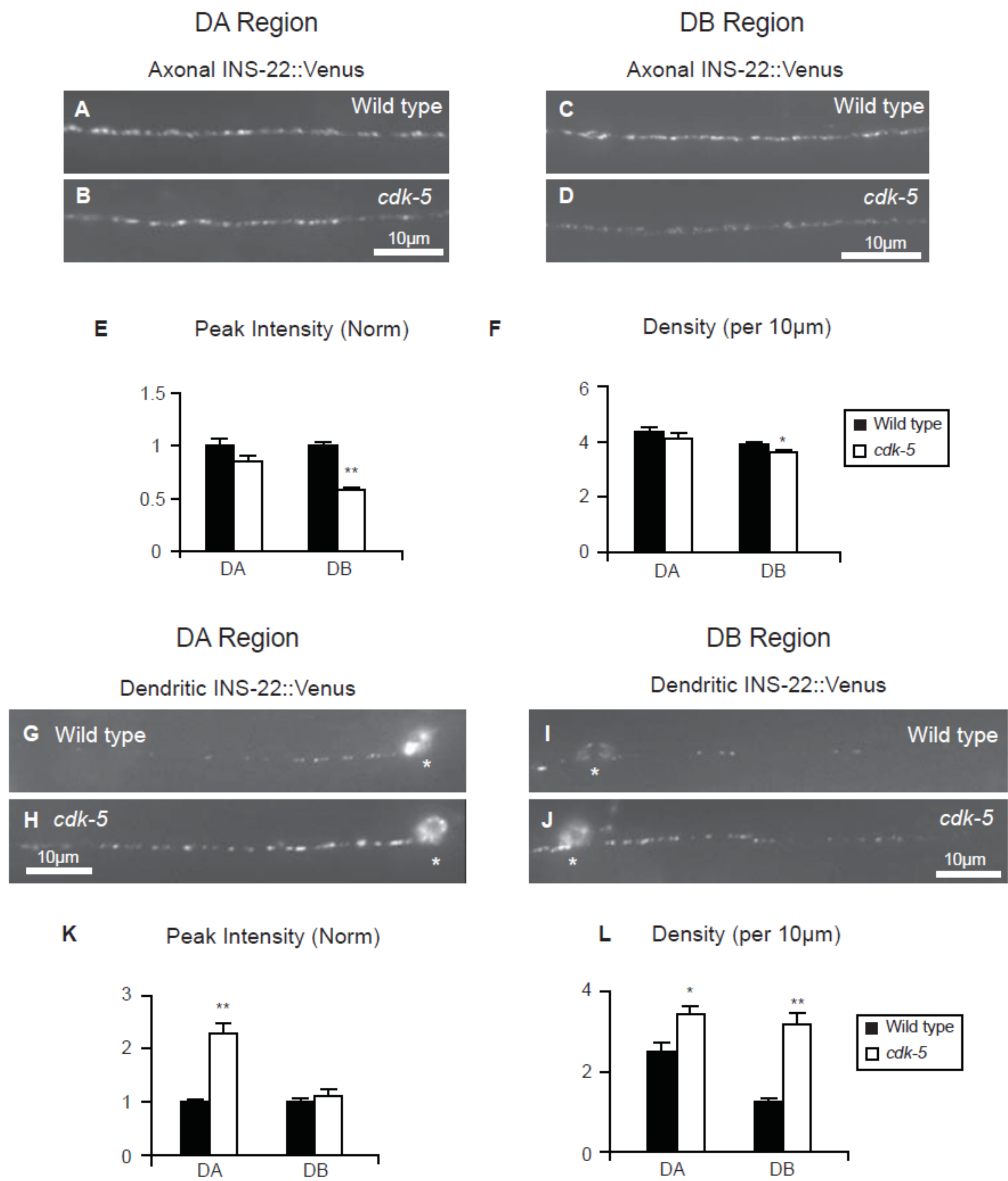

Figure 3-2. CDK-5 promotes axonal localization of DCVs in DB type motor neurons only, but inhibits dendritic localization in both DA and DB type motor neurons. (**A-B**) Representative images of INS-22::Venus localization in DA axons of wild-type (**A**) and *cdk-5(gm336)* mutant (**B**) animals. (**C-D**) Representative images of INS-22::Venus localization in DB axons of wild-type (**C**) and *cdk-5(gm336)* mutant (**C**) animals. (**E-F**) Quantitative fluorescence analysis of normalized INS-22::Venus puncta intensity (**E**) and density (**F**) in the DA axons of wild-type (n=20) and *cdk-5* mutant animals (n=22) and DB axons of wild-type (n=101) and *cdk-5(gm336*) (n=46) mutants (**G-H**) Representative images of INS-22::Venus localization in DA dendrites of wild-type (**G**) and *cdk-5(gm336)* mutant (**H**) animals. (**I-J**) Representative images of INS-22::Venus localization in DB dendrites of wild-type (**I**) and *cdk-5(gm336)* mutant (**J**) animals. (**K-L**) Quantitative fluorescence analysis of INS-22::Venus puncta intensity (**K**) and (**L**) in DA dendrites of wild-type (n=19) and *cdk-5* (n=20) and DB dendrites of wild type (n=27) and *cdk-5* (n=19). Values that differ significantly from wild type are indicated by asterisk (*p<0.01, **p<0.001).

To achieve stricter separation between changes in DA neurons and DB neurons, we performed time-lapse analysis of mobile INS-22::Venus in DA and DB commissures. The commissure is a curved section of the DA/DB motor neuron axon which connects the cell body to the axon in the dorsal nerve cord (Fig. 3-1). Although the curved morphology of the commissure prevents accurate analysis of DCV velocities or run lengths, we can image DCVs moving within a single DA or DB commissure and determine if they are moving anterogradely or retrogradely. When we compared INS-22::Venus movement in the DA3 and DB6 commissure, we again observed a DB-specific effect of *cdk-5* mutation (Fig. 3-3 A-C). The number of anterograde puncta, retrograde puncta, and stationary puncta were not significantly different in *cdk-5* mutants compared to wild-type animals in the DA3 commissure (Fig. 3-3 C). In contrast, *cdk-5* mutants had significantly more stationary INS-22::Venus puncta compared to wild type controls in the DB6 commissure (Fig. 3-3 F, $p<0.001$). *cdk-5* mutants also had a significant ($p<0.05$) decrease in the amount of anterograde trafficking in the DB6 commissure relative to wild-type animals. These findings are consistent our static imaging results of presynaptic INS-22::Venus in the DNC and suggest that CDK-5 promotes anterograde DCV movement in DB neurons only.

Regulation of axonal DCV trafficking by CDK-5 and CYY-1

Because we saw no change in *cdk-5* mutant trafficking of DCVs in DA axons, we wondered whether PCT-1/PCTAIRE kinase activity can compensate for the loss of *cdk-5* in DA neurons. Although Ou et al (2010) do not quantitate RAB-3::GFP levels in the axon, they show that *cdk-5* and *cyy-1* single mutants visible SV localization to the axon. In contrast, mutation of both genes together results in a dramatic loss of SVs from the axon, suggesting that each kinase can compensate for the loss of the other (Ou et al., 2010). Furthermore, the authors demonstrate that *cdk-5;cyy-1* mutants have increased retrograde trafficking of

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FIGURE 3

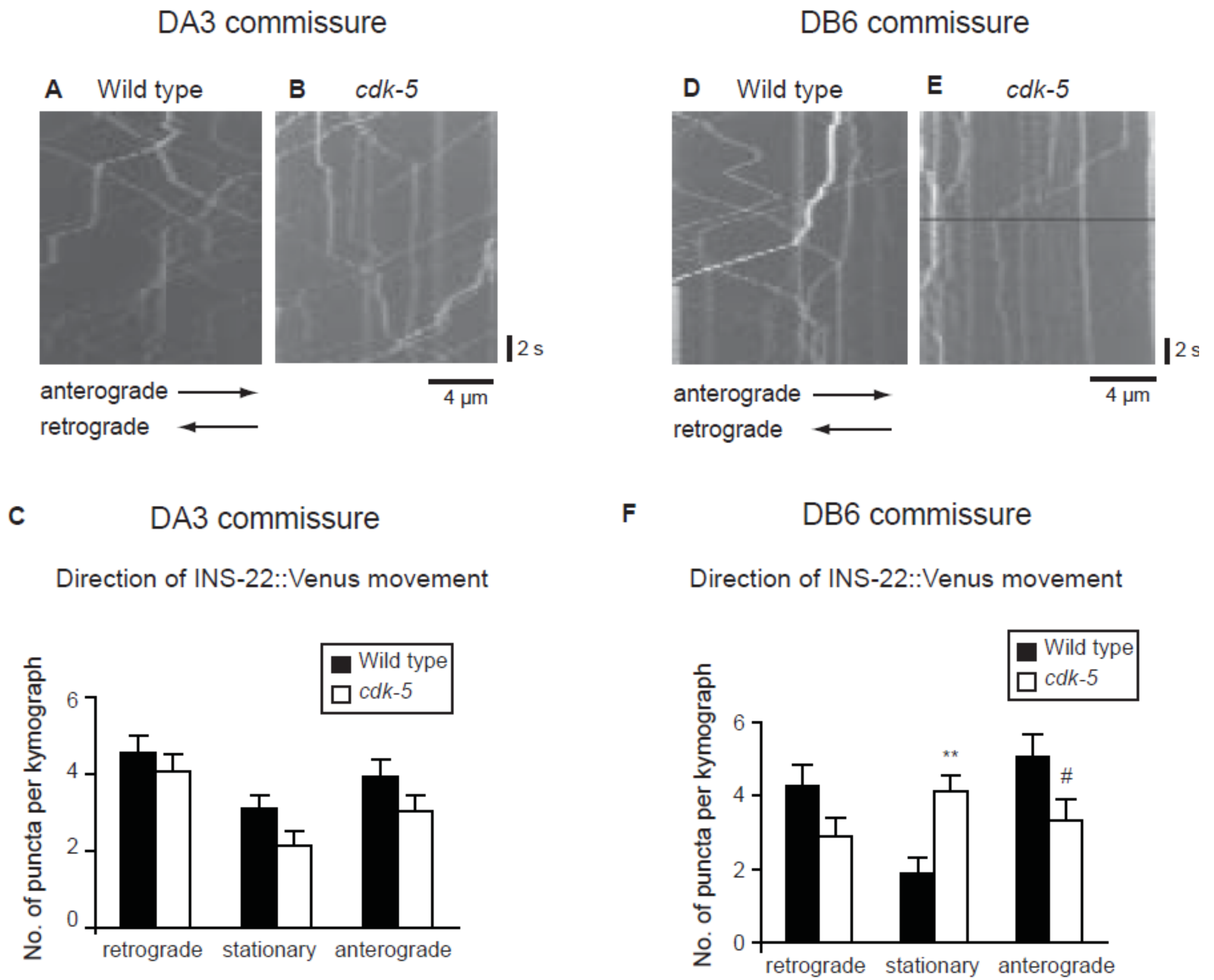

Figure 3-3. CDK-5 regulates anterograde DCV mobility in the axon of DB neurons only. (**A-B**) Kymographs generated from 20 second movies of mobile INS-22::Venus puncta in the DA3 commissure of wild-type (**A**) and *cdk-5(gm336)* (**B**) mutant animals. (**C**) Quantification of the number of anterograde, retrograde, and stationary INS-22::Venus puncta in DA3 commissure of wild-type (n=29) and *cdk-5* mutant (n=30) animals. (**D-E**) Kymographs generated from 20 second movies of mobile INS-22::Venus puncta in the DB6 commissure of wild-type (**D**) and *cdk-5(gm336)* (**E**) animals. (**F**) Quantification of the number of anterograde, retrograde, and stationary INS-22::Venus puncta in the DB6 commissure of wild-type (n=23) and *cdk-5* mutant (n=25) animals. Values that differ significantly from wild type are indicated by (**$p<0.001$, #$p<0.05$).

SVs in the axon, and that this increase is eliminated in dynein heavy chain (*dhc-1)* mutants. We tested whether DCV localization might be regulated in a similar way by examining the localization of INS-22::Venus in animals with mutations in PCT-1's activator, *cyy-1.*

Similar to *cdk-5(gm336)* mutants, *cyy-1(wy302)* single mutants had no change in axonal INS-22::Venus in DA neurons relative to wild-type animals (Fig. 3-4 A-C). In the *cdk-5(gm336);cyy-1(wy302)* double mutants, however, we observed a synthetic effect of the two mutations. *Cdk-5;cyy-1* double mutants had significantly less INS-22::Venus fluorescence than wild type animals or either single mutant (Fig. 3-4 A-F). These results demonstrate that CDK-5 and CYY-1 act in redundant pathways to regulate the abundance of DCVs in the axons DA neurons. Furthermore, these results suggest that CDK-5 and CYY-1 regulate DCV and SV localization through similar mechanisms in DA motor neurons.

We next tested whether mutation of *cyy-1* would enhance the decrease in axonal DCV abundance in *cdk-5* mutant DB neurons. As described above, *cdk-5(gm336)* mutants have decreased INS-22::Venus puncta fluorescence in the DB axons. However, unlike *cdk-5(gm336)* mutants, *cyy-1(wy302)* mutants had no significant decrease in INS-22::Venus puncta intensity in the DB axon relative to wild-type animals (Fig. 3-4 G-I). There was, however, a small but significant decrease in INS-22::Venus density in DB axons in *cyy-1* mutants. When we examined INS-22::Venus in DB axons of *cdk-5;cyy-1* double mutants, we found that *cyy-1* mutations did not enhance the decrease in INS-22::Venus fluorescence observed in *cdk-5* single mutants (Fig. 3-4 G-L). *cdk-5;cyy-1* double mutants had the same level of INS-22::Venus puncta fluorescence in the axon as *cdk-5* single mutants (Fig. 3-4 K) and the same density of INS-22::Venus puncta as *cyy-1* single mutants (Fig. 3-4 L). Thus, we found no synthetic effect between the *cdk-5 and cyy-1* mutations in DB axons, as we had observed in DA. These findings indicate that, in contrast to what we found in DA neurons,

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FIGURE 4

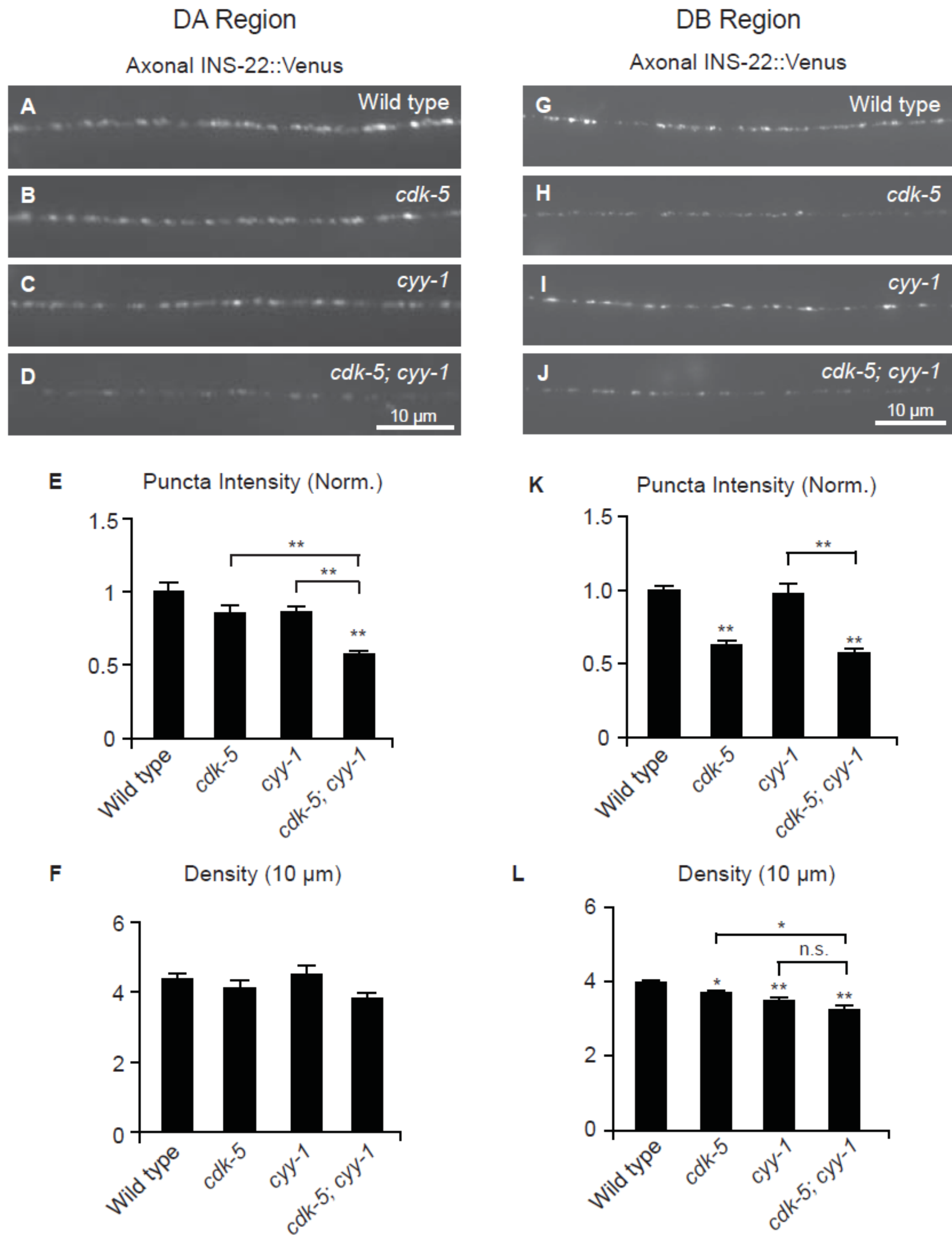

Figure 3-4. CDK-5 and CYY-1 regulate DCV localization to DA axons redundantly (**A-D**) Representative images of INS-22::Venus in wild-type (**A**), *cdk-5(gm336)* (**B**), *cyy-1(wy302)* (**C**), and *cdk-5;cyy-1* double mutants (**D**). (**E-F**) Quantitative fluorescence analysis of INS-22::Venus puncta intensity (**E**) and density (**F**) in the DA axons of wild type (n=20), *cdk-5* (n=22), *cyy-1*(n=17), and *cdk-5;cyy-1* (n=19) animals. (**G-J**) Representative images of axonal INS-22::Venus in DB neurons of wild type (**G**), *cdk-5(gm336)* (**H**), *cyy-1*(**I**), and *cdk-5; cyy-1* double (**J**) mutant animals. (**K-L**) Quantitative fluorescence analysis of INS-22::Venus puncta intensity (**K**) and density (**L**) in DB axons of wild type (n=101), *cdk-5*(n=46), *cyy-1*(n=23), and *cdk-5;cyy-1*(n=23) animals. Values that differ significantly are indicated by asterisk (#p<0.05, *p<0.01, **p<0.001).

CDK-5 and CYY-1 do not act in redundant pathways to regulate axonal localization of DCVs in DB neurons.

Collectively, these results suggest that CDK-5 can regulate DCV abundance in the axon through a mechanism that (1) is specific to DB neurons (2) is separable from the mechanism of inhibition of dendritic trafficking, and (3) is different from regulation by the PCT-1/CYY-1 pathway.

CDK-5 regulates INS-22::Venus release from axons

One possible explanation for the observed decrease in INS-22::Venus in the DB axons of *cdk-5* mutants, in addition to changes in axonal trafficking, is that *cdk-5* mutants have enhanced neuropeptide release. Because INS-22::Venus is packaged into the lumen of DCVs (Sieburth et al., 2007; Speese et al., 2007) mutations that enhance DCV release can decrease INS-22::Venus fluorescence at presynaptic sites, and, conversely, mutations that decrease DCV release can increase INS-22::Venus at presynaptic sites (Ch'ng et al., 2008; Gracheva et al., 2006; Sieburth et al., 2007).

To determine if decreased INS-22::Venus fluorescence in *cdk-5* mutant axons could be attributed to increased DCV release, we blocked DCV release in *cdk-5(gm336)* mutants by crossing into the *unc-31(e978)/CAPS* mutant background. UNC-31/CAPS is a Ca^{2+}-sensing protein that is required for DCV docking and *unc-31* mutations result in DCV accumulation in the DNC and reduced neuropeptide release (Hammarlund et al., 2008; Sieburth et al., 2007; Speese et al., 2007). By preventing release of DCVs with an *unc-31* mutation, we can eliminate the effect of neuropeptide release on INS-22::Venus signal in the axon and isolate the effect of *cdk-5* on DCV trafficking. In agreement with previous studies of *unc-31* (Ch'ng et al., 2008), we found that INS-22::Venus fluorescence intensity was significantly increased in DB axons of *unc-31(e978)* mutant animals relative to wild type

controls, consistent with decreased DCV release (Fig. 3-5 G,I, and K). Surprisingly, *unc-31* mutations completely blocked the decrease in INS-22::Venus observed in *cdk-5* single mutants (Fig. 3-5 J-L). These results suggest that the loss of INS-22::Venus in *cdk-5* mutant axons may not only be attributed to less trafficking of DCVs, but also to increased release of INS-22::Venus from DCVs in DB axons.

Complementary to these results, we found that INS-22::Venus abundance in DA neurons is not regulated by UNC-31. *unc-31(e978)* mutants did not have significantly increased INS-22::Venus fluorescence intensity in DA neurons and *cdk-5;unc-31* double mutants were also not significantly different from wild-type animals (Fig. 3-5 A-F). These results are consistent with the idea that CDK-5 is a negative regulator of UNC-31-dependent DCV release. In neurons where UNC-31 does not play a key role in regulation of INS-22::Venus fluorescence (i.e. DA neurons), CDK-5 also does not play a role in regulation of INS-22::Venus fluorescence.

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FIGURE 5

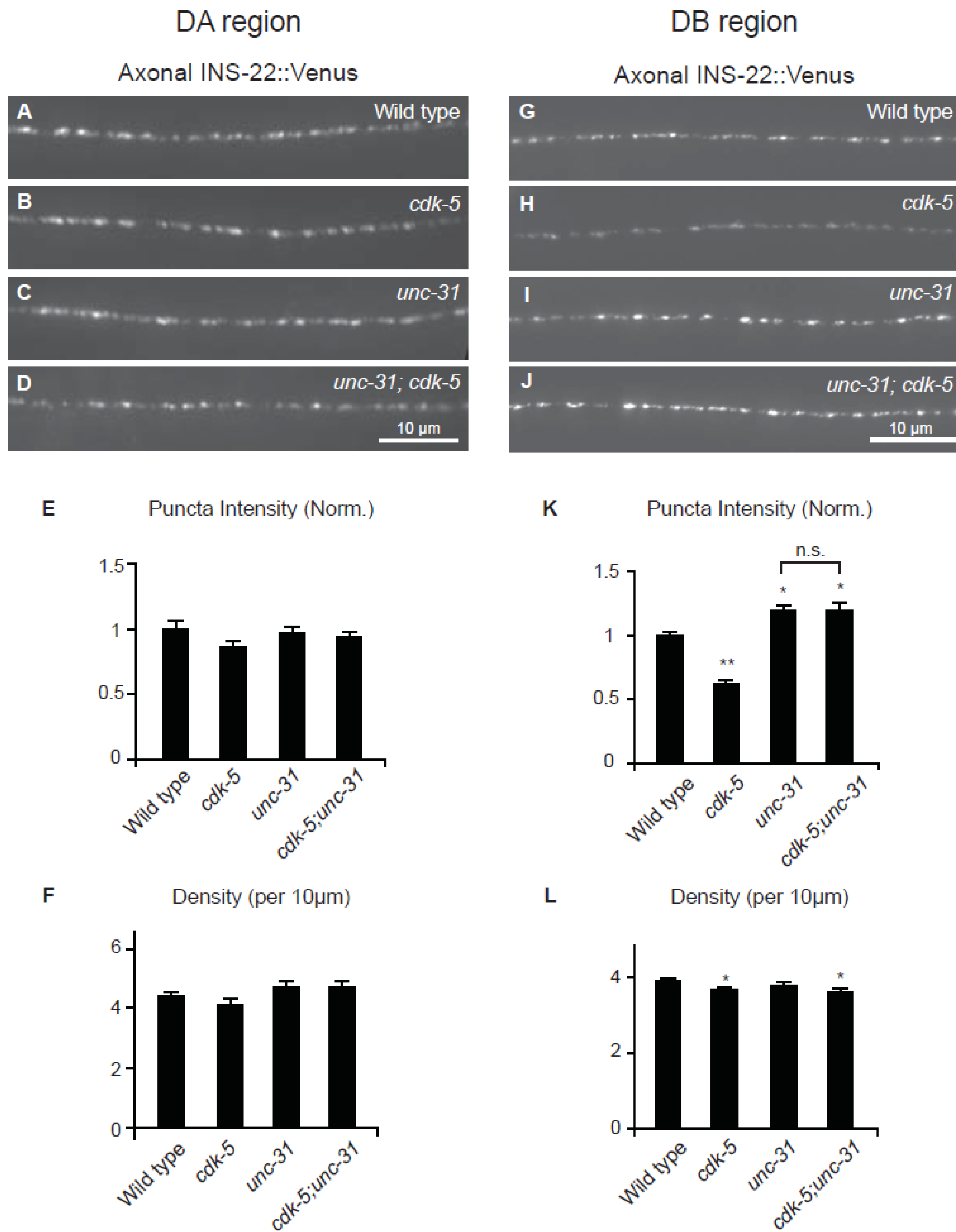

Figure 3-5. CDK-5 negatively regulates DCV release in DB neurons (**A-D**)

Representative images of INS-22::Venus in wild-type (**A**), *cdk-5(gm336)* (**B**), *unc-31(e978)* (**C**), and *cdk-5;unc-31* double mutants (**D**). (**E-F**) Quantitative fluorescence analysis of INS-22::Venus puncta intensity (**E**) and density (**F**) in the DA axons of wild type (n=20), *cdk-5* (n=22), *unc-31*(n=19), and *cdk-5;unc-31* (n=20) animals. (**G-J**) Representative images of axonal INS-22::Venus in DB neurons of wild type (**G**), *cdk-5(gm336)* (**H**), *unc-31(e978)*(**I**), and *cdk-5;unc-31* double (**J**) mutant animals. (**K-L**) Quantitative fluorescence analysis of INS-22::Venus puncta intensity (**K**) and density (**L**) in DB axons of wild type (n=101), *cdk-5*(n=46), *unc-31*(n=27), and *cdk-5;unc-31*(n=22) animals. Values that differ significantly are indicated by asterisk (#p<0.05, *p≤0.01, **p<0.001).

Discussion

In this chapter, we show that CDK-5 regulates the abundance of axonal DCVs in a cell-type specific manner. The fluorescence of a DCV marker, INS-22::Venus, is decreased in *cdk-5* mutants in the axons of DB-type motor neurons, but not in the axons of DA-type motor neurons. This cell-type specific effect in the axon appears to be different from CDK-5's role in the dendrite. Dendritic DCVs are increased by *cdk-5* mutation in both DA and DB cell types. These results show that CDK-5 can inhibit dendritic localization of DCVs without promoting axonal localization at the same time.

The lack of change in the axons of DA neurons in *cdk-5* mutants can be attributed to compensation by another member of the cyclin-dependent kinase family, PCT-1 kinase and its activator CYY-1. In has recently been shown that overexpression of the cyclin-dependent kinase family member *pct-1* can compensate for loss of *cdk-5* function and correct defects in the polarized trafficking of SVs seen in *cdk-5* mutants (Ou et al., 2010). Consistent with this previous report, we saw that DCV localization in the DA axon was not disrupted in *cdk-5* single mutants, but was disrupted in *cdk-5*;*cyy-1* double mutants (Fig. 3-4). This result suggests that the CYY-1 pathway can compensate for the loss of CDK-5 in DA neurons. Together, these results imply that DCV and SV trafficking are regulated by similar mechanisms in DA neurons, where CDK-5 and PCT-1 kinase function in parallel, redundant pathways.

In DB neurons, however, CDK-5 plays a role in axonal localization of DCVs for which PCT-1 cannot compensate. *cdk-5* single mutants have less INS-22::Venus marker in DB axons than wild-type controls, demonstrating that CDK-5 regulates the amount of neuropeptides in the DB axon (Fig. 3-2). We found evidence in support of two potential mechanisms by which CDK-5 may promote axonal localization of neuropeptides. First, we found that *cdk-5* mutants have alterations in the trafficking of DCVs to the axon. *cdk-5*

mutants have more stationary INS-22::Venus puncta in the axon and fewer anterograde INS-22::Venus puncta compared to wild-type animals (Fig. 3-3), suggesting that CDK-5 promotes anterograde trafficking of DCVs to the axon. Second, we used epistasis experiments to test if the decrease in INS-22::Venus in the axon could also be attributed to enhanced neuropeptide release. When DCV release was blocked by mutations in *unc-31/CAPS*, we could no longer see a *cdk-5*-dependent effect on INS-22::Venus fluorescence in the axon (i.e. *unc-31* single mutants and *unc-31;cdk-5* double mutants were the same, Fig. 3-5), suggesting that INS-22::Venus fluorescence decreased in *cdk-5* mutants in part because more peptides were released. These results are consistent with a model where CDK-5 inhibits UNC-31-dependent release of DCVs in DB axons. CDK-5's regulation of neuropeptide release may have stronger effects on DCV localization to the axon than the changes in trafficking we observed, because blocking peptide release completely eliminates the *cdk-5*-dependent decreases in INS-22::Venus in the axon (Fig. 3-5). However, we cannot rule out the possibility that the alterations we observe in trafficking also contribute to decreased axonal INS-22::Venus fluorescence, and further studies will be necessary to discriminate between these two possibilities or determine if both mechanisms are at work.

CDK-5 regulates axonal trafficking of DCVs in DB neurons

In Chapter 2, we described a role for CDK-5 in inhibition of anterograde trafficking of DCVs to the dendrite. This begged the question of whether the changes we observed in axonal trafficking are ultimately caused by the changes in dendritic trafficking. An increase in dendritic trafficking might be predicted to cause less axonal trafficking if re-direction of DCVs to the dendrite leaves too few vesicles available for transport into the axon. However, two lines of evidence make this unlikely. First, *cdk-5* mutants have a robust increase in dendritic DCVs without any loss of DCVs from the axon (Fig. 3-2) or a decrease in anterograde DCV

trafficking in the commissure (Fig. 3-3) in DA neurons. Second, in DB axons we found a decrease in anterograde trafficking of DCVs and also a significant increase in stationary DCVs (Fig. 3-3). Although decreased anterograde trafficking could be ascribed to a lack of DCVs available for axonal transport, this would not explain why there are more stationary DCVs in the commissure.

Our finding that CDK-5 regulates axonal anterograde trafficking of DCVs in DB axons is consistent with previous studies of CDK-5's role in regulation of axonal transport of membrane-bound organelles in squid giant axons (Morfini et al., 2004). Inhibition of CDK-5 activity reduces the velocity of anterograde transport, without affecting retrograde transport, and also reduces the association of Kif5 (kinesin-1) with its cargoes (Morfini et al., 2004). Similarly, we found less anterograde transport of DCVs in *cdk-5* mutants and no change in the amount of retrograde transport (Fig. 3-3). We also observed more stationary DCVs in DB axons, consistent with decreased motor-cargo association. This increase in stationary DCVs was also seen in the dendrite of *cdk-5* mutants (Chapter 2, Fig. 2-3), which raises the possibility that CDK-5 may be required for DCVs to remain mobile. The fact that we do not see any changes in retrograde trafficking is intriguing, considering that dynein was implicated in the increased trafficking of DCVs (Chapter 2) and SVs (Ou et al., 2010) to the dendrite in *cdk-5* mutants. If a similar mechanism were functioning in the axon, we would have expected to see an increase in retrograde trafficking rather than a decrease in anterograde trafficking.

CDK-5 inhibits neuropeptide release from DB neurons

Multiple groups have shown that CDK-5 negatively regulates vesicle release in a variety of cell systems (Angelo et al., 2006). CDK-5 negatively regulates the size of the releasable pool and increases the size of the resting pool in hippocampal neurons (Kim and

Ryan, 2010) and also negatively regulates release of dopamine from the striatum (Chergui et al., 2004). CDK-5 activity reduces quantal size and narrows the kinetics of vesicle fusion in adrenal chromaffin cells (Barclay et al., 2004). In pancreatic beta cells, CDK-5 negatively regulates insulin release from DCVs in response to high glucose (Wei et al., 2005). CDK-5 has also been shown to phosphorylate SEPT5, causing SEPT5 to bind syntaxin and prevent vesicle release in PC12 cells (Amin et al., 2008).

In this study, we found evidence for a role for CDK-5 in the negative regulation of neuropeptide release. The CDK-5-dependent effect on DCV localization in the axon was eliminated by blocking DCV release with *unc-31* mutations (Fig. 3-5). *cdk-5;unc-31* double mutants and *unc-31* single mutants have similar levels of INS-22::Venus abundance in DB axons. It is possible that these two genotypes have similar amounts of INS-22::Venus in DB axons because there is a ceiling effect on DCV accumulation at presynaptic sites, and both genotypes have reached the ceiling, which obscures any differences between the two genotypes. However, it is unlikely *unc-31* mutants have reached a ceiling for DCV accumulation. Our data and data from a previously published paper (Ch'ng et al., 2008) indicate that *unc-31* mutations cause a 18-20% increase in INS-22::Venus fluorescence in the DNC, but mutation of the syntaxin-binding protein *unc-13* can cause a 32% increase in INS-22::Venus fluorescence (Ch'ng et al., 2008). Thus, the accumulation of DCVs in *unc-31* and *cdk-5;unc-31* mutants has not reached the maximum amount of DCV accumulation that can occur at presynaptic sites.

Unexpectedly, we also observed that *unc-31* mutations did not lead to INS-22::Venus accumulation in DA neurons (Fig. 3-5). This finding complemented our results from DB neurons; only in axons of cell types dependent on UNC-31 for DCV release do we observe *cdk-5*-dependent changes in INS-22::Venus fluorescence. DA neurons may compensate for loss of *unc-31* by using other mechanisms of DCV release. In fact, *unc-13* has also been

shown to promote DCV docking (Hammarlund et al., 2008) and PKA activation can fully rescue defects in DCV docking in *unc-31* mutants (Zhou et al., 2007). These studies suggest that DA neurons may release DCVs through an alternate pathway. It will be interesting to see if CDK-5 plays a general role in inhibition of neuropeptide and neurotrophin release, or if its effects are specific for either UNC-31-mediated release or release of INS-22. Further studies will be needed to determine if any of the previously described CDK-5 substrates are involved in the regulation of INS-22 release.

Dual roles of CDK-5

Both increased neuropeptide release and decreased DCV trafficking can potentially contribute to the large decrease in neuropeptide localization in the axon that occurs in *cdk-5* mutants. Taken together, these two effects may explain why, in the previous chapter, we observed a small decrease in the membrane-bound DCV marker IDA-1::GFP in *cdk-5* mutant axons that was not as strong as the decrease seen with INS-22::Venus (Chapter 2, Fig. 2-S1). Lumenal markers, such as INS-22::Venus, will diffuse away after release and be taken up into coelomocyte scavenger cells (Sieburth et al., 2007; Speese et al., 2007). Therefore, INS-22::Venus fluorescence in axons varies depending on the rate of DCV trafficking and release. DCV membrane proteins, such as IDA-1::GFP, on the other hand, fuse briefly with the plasma membrane during exocytosis and then are subsequently recaptured from the plasma membrane (Taraska et al., 2003). Because IDA-1::GFP was presumably only affected by trafficking but not release, we only observed a small (albeit) significant decrease in IDA-1::GFP in DB axons *cdk-5* mutants.

The finding that *cdk-5* mutants have increased neuropeptide release in DB neurons raises the question of whether the increases in dendritic DCVs observed in DB neurons are a secondary effect of increased release. If feed-back mechanisms exist to keep the amount

of DCVs at release sites in the axon above a set point, the neuron may up-regulate DCV biosynthesis and trafficking, leading to extra trafficking of DCVs into the dendrite. However, we think this model is less likely to be true because we observed robust increases in dendritic DCVs in the DA neurons of *cdk-5* despite the fact that there was no change in INS-22::Venus abundance (or release) in the axons.

Another way in which increased DCV release could indirectly alter trafficking is by increasing neuronal firing rates. Release of neuropeptides can positively regulate synaptic transmission in *C. elegans* (Jacob and Kaplan, 2003; Sieburth et al., 2005). Depolarization of mammalian neurons decreases the velocity of DCVs carrying Sema3a and increases the number of stationary DCVs in axons (de Wit et al., 2006). Greater neuropeptide release could potentially enhance neuronal activity and lead to changes in DCV movement. Further characterization of other genetic mutants with altered DCV release and synaptic activity will be necessary to fully understand the relationship between neuropeptide release and DCV trafficking dynamics.

Materials and Methods:

Strains

The following strains were used in this study: nuIs195 [Punc129::INS-22::Venus], *cdk-5(gm336), cyy-1(wy302), unc-31(e978).* The *wy032* allele corresponds to a premature stop codon at amino acid 6 and is likely a null mutant.

Static Imaging

Basic imaging techniques were used as described in Chapter 2. For DB neuron axon imaging, images were taken near the posterior bend of the gonad. The *Punc129* promoter drives expression in 3 motor neurons in this area of the cord – DA6, DB6, and DB7. DA6 and DB6 have overlapping, oppositely projecting axons in the anterior third of this area and everything posterior to this area of overlap contains signal from DB neurons only. Thus, these images are enriched with INS-22::Venus signal from DB neurons. For DA motor neuron axon imaging, images were taken in the anterior portion of the DNC adjacent to the pharynx. This section of the DNC contains signal from DA3, DA2 and DB2 axons. In wild type animals, INS-22::Venus is only visible in the DB2 cell body in ~20% of animals, suggesting the *Punc129* promoter does not drive expression strongly in this neuron, and that most of the INS-22::Venus signal comes from DA neurons. For ventral nerve cord images (dendrites), line scans were drawn along DA3 or DB6 processes. Line scans of nerve cord puncta were generated using MetaMorph (v6.0) and were then analyzed in Igor Pro (v5) using custom written software as previously described (Burbea et al., 2002). Puncta intensity and density were calculated from each image. Puncta intensity is the fractional increase in peak fluorescence of each puncta over fluorescent bead intensity for that date. Puncta density is the average number of puncta per 10 μm of nerve cord.

Time-lapse imaging

For time-lapse imaging, animals were paralyzed using levamisol, as described in Chapter 2. Time-lapse imaging of mobile INS-22::Venus puncta was performed in the DA3 commissure and DB6 commissure. 20 second movies of INS-22::Venus movement in the commissure was recorded at a 4 Hz speed. Line scans were traced from the DA3 or DB6 cell body towards the DNC for each time lapse movie and these scans were used to generate kymographs. Puncta movement was analyzed in the kymograph. Each puncta was designated as mobile if its velocity exceeded 0.1um/sec and it moved greater than twice its own width. Data were compiled in Microsoft Excel for analysis and Student's T test was performed to determine differences between genotypes.

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CHAPTER 4

Cylcin-dependent kinase-5 positively regulates synaptic transmission at the *C. elegans* neuromuscular junction

Attributes: Experiments in the following chapter were performed by Patricia Goodwin, Marianna Pappageorge, and Peter Juo. Experiments from Figures 2 through 5 were performed by Patricia Goodwin. Figure 1 and Figure 6 A-F were drawn/performed by Peter Juo. Figure 6G was performed by Marianna Papageorge.

Introduction

Cyclin-dependent kinase 5 is a kinase with many roles in the regulation of neuronal development, synaptic transmission, plasticity, and neurodegeneration. Inactivation of CDK-5 has been shown to be neuroprotective in animal models of many neurodegenerative diseases, including Huntington's Disease (HD), prion protein disease, and Alzheimer's Disease (AD)(Cruz and Tsai, 2004; Lopes et al., 2007; Paoletti et al., 2008). While a large body of research describes CDK-5's induction of neurofibrillary tangle formation (Crews and Masliah, 2010; Tsai et al., 2004), CDK-5's role in regulation of synaptic transmission may also be relevant to understanding AD progression. Loss of synapses has been shown to be a better correlate of cognitive decline in patients with AD than plaque formation or tangles (Coleman and Yao, 2003). Loss of cholinergic neurotransmission in particular can affect memory and treatments that enhance cholinergic transmission can temporarily improve cognitive performance in patients with AD (Pepeu and Giovannini, 2010). Characterization of CDK-5's substrates at the synapse could potentially lead to better understanding of its role in neurodegenerative disease progression.

CDK-5 activity can regulate synaptic morphology, postsynaptic neurotransmitter receptor clustering and function, and presynaptic exocytosis and endocytosis (Dhavan and Tsai, 2001). In mice and *Drosophila*, *cdk-5* null animals have aberrant branching of motor neuron axons at the NMJ (Fu et al., 2005; Kissler et al., 2009). CDK-5 also promotes active zone assembly through phosphorylation of the MAGUK family member CASK, which increases CASK recruitment to synapses (Samuels et al., 2007). Dendritic spine formation has been shown to be both positively and negatively regulated by CDK-5 (Angelo, 2006; Lai, 2009). Postsynaptically, CDK-5 regulates the localization and function of NMDA, AMPA, and acetylcholine receptors (AChR)(Angelo et al., 2006; Hawasli and Bibb, 2007). *Cdk-5* knock-out mice have increased agrin-dependent AChR clustering (Fu et al., 2005). In *C.*

elegans, CDK-5 is required for the localization of the AMPA-type receptor GLR-1 to synapses (Juo et al., 2007). CDK-5 has opposing roles on NMDA receptor stability at the synapse and ion conductance; CDK-5 activity increases receptor degradation and reduces PSD-95 clustering, but also increases NMDA receptor channel conductance (Angelo et al., 2006; Lai and Ip, 2009).

On the presynaptic side, CDK-5 has been shown to regulate both synaptic vesicle release and endocytosis. CDK-5 activity regulates the release of synaptic vesicles, insulin-containing vesicles, and chromaffin granules (Angelo et al., 2006; Dhariwala and Rajadhyaksha, 2008; Dhavan and Tsai, 2001; Hawasli and Bibb, 2007). CDK-5 has been shown to both positively and negatively regulate insulin secretion. At low glucose levels, CDK-5 stimulates the release of insulin from pancreatic beta cells by phosphorylating Munc-18 (Lilja et al., 2004; Lilja et al., 2001), but at high levels of glucose it acts as an inhibitor of insulin secretion (Wei et al., 2005). CDK-5 activity can limit vesicle secretion through phosphorylation of septin 5 (SEPT5), which decreases its binding to the SNARE protein syntaxin-1 (Amin et al., 2008). CDK-5 has also been shown to inhibit synaptic transmission in hippocampal neurons by increasing the percent synaptic vesicles in the resting pool (Kim and Ryan, 2010). Early studies using CDK-5 inhibitors implicated CDK-5 in regulation of P/Q-type calcium channel conductance, but these results may be due in part to off-target effects of CDK-5 inhibitors (Angelo et al., 2006; Hawasli and Bibb, 2007; Tomizawa et al., 2002). Additionally, CDK-5 has been shown to phosphorylate components of the endocytic machinery, including dynamin and amphiphysin, leading to decreased endocytosis (Tomizawa et al., 2003).

Although multiple CDK-5 targets have been identified at the synapse, identification of up-stream regulators of CDK-5 activity has lagged behind. Studies of CDK-5 have proven challenging in general, because mouse *cdk5* nulls are embryonic lethal (Dhavan and Tsai,

2001), small molecule inhibitors of CDK-5 have off-target effects (Angelo et al., 2006; Hawasli and Bibb, 2007), and there is no clear consensus sequence for CDK-5 substrates apart from an up-stream proline (Dhariwala and Rajadhyaksha, 2008; Dhavan and Tsai, 2001). A more genetically tractable model system could therefore prove valuable for identifying CDK-5 substrates and regulators.

The model organism *C. elegans* is a potentially useful tool for the study of CDK-5's role in the regulation of synaptic transmission. Genetic null mutants of *cdk-5* and CDK-5's activator, *cdka-1*/*p35* are viable in *C. elegans* (Juo et al., 2007). *C. elegans* are amenable to forward genetic screening, genome-wide RNAi, and candidate gene screening (Kamath et al., 2003), which could be used to identify new CDK-5 substrates and new upstream CDK-5 regulators. *C. elegans* has a simple nervous system, with only 302 neurons. Additionally, because there is less gene redundancy in *C. elegans*, gene knock-down is less likely to result in compensatory up-regulation of redundant genes (Cavalcanti et al., 2003; Gu et al., 2002). Many genes that are critical for synaptic function can be mutated or eliminated in *C. elegans* without producing embryonic lethality (Miller et al., 1996b; Nguyen et al., 1995), making genetic epistasis experiments with these mutants and *cdk-5* mutants possible. Finally, a simple behavioral assay called the aldicarb assay can be used in *C. elegans* to indirectly measure synaptic transmission at the neuromuscular junction (NMJ) (Miller et al., 1996a; Nguyen et al., 1995; Sieburth et al., 2005).

Aldicarb is an acetylcholinesterase (AChE) inhibitor that prevents the break-down of acetylcholine (ACh) in the synaptic cleft. This leads to increased ACh levels in the synaptic cleft of the NMJ, which causes activation of postsynaptic AChRs on muscle cells, leading to hyper-contraction of the body muscles and rigid paralysis (Miller et al., 1996a; Nguyen et al., 1995; Sieburth et al., 2005)(Fig. 4-1 A). The latency to paralysis depends on the amount of acetylcholine released, the expression and sensitivity of AChRs in the muscle, and the

CHAPTER 4
FIGURE 1

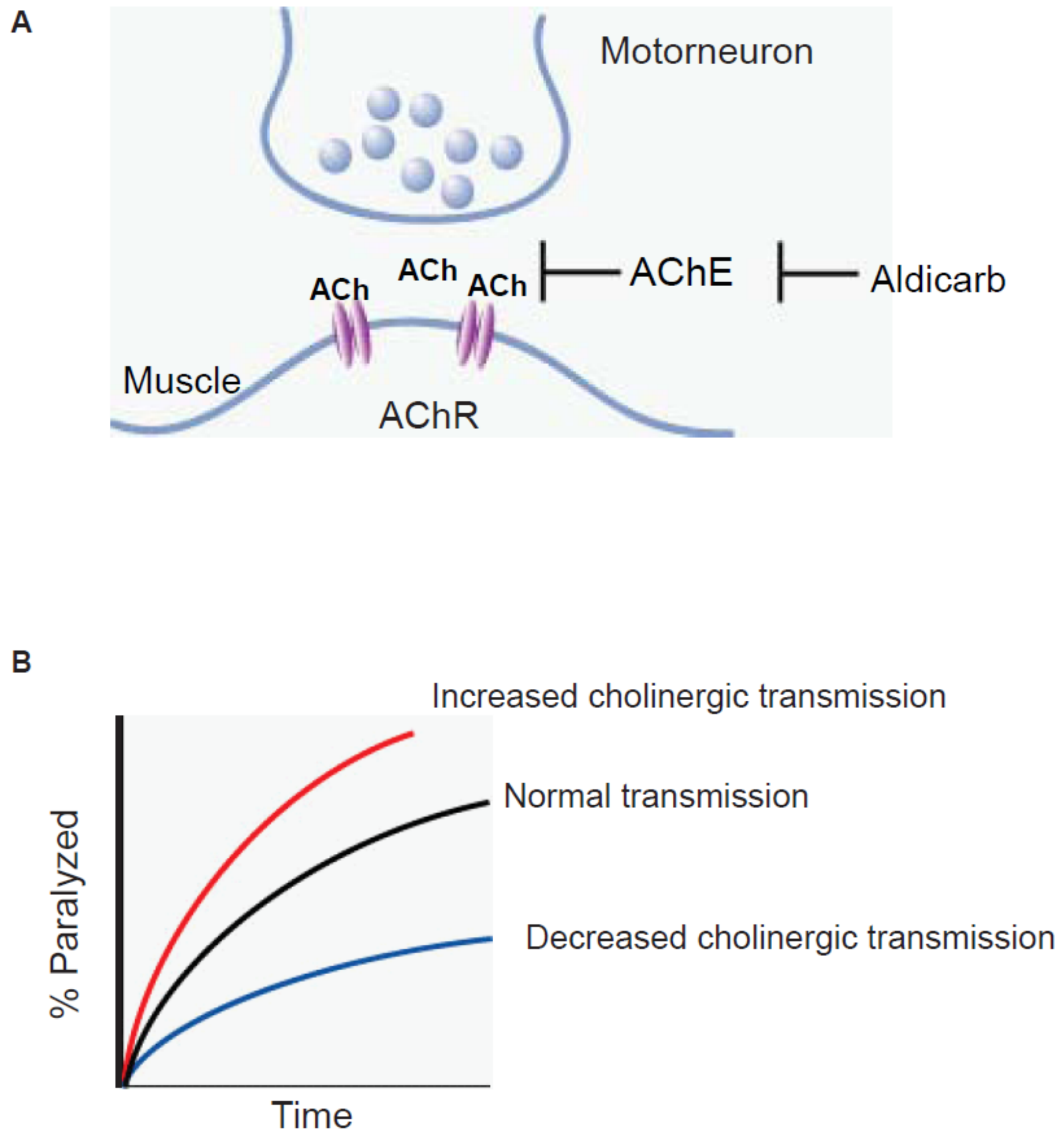

Figure 4-1. Aldicarb-induced paralysis. (**A**) Following release of acetylcholine (ACh) from the presynaptic motor neuron, ACh is broken down in the synaptic cleft by acetylcholinesterase (AChE). Aldicarb is an inhibitor of acetylcholinesterase, therefore treatment with aldicarb increases ACh levels in the synaptic cleft. Increased ACh in the synaptic cleft activates postsynaptic ACh receptors (AChR), leading to hyper-contraction of the muscles and paralysis. (**B**) Example graph of time-dependent aldicarb paralysis for animals with normal transmission, animals with decreased cholinergic transmission, and animals with increased cholinergic transmission. Paralysis is measured as the percent of animals that do not respond to prodding at a given time-point. Animals with decreased cholinergic transmission have reduced build-up of ACh in the synaptic cleft or reduced activation of AChR, so fewer worms paralyze over time compared to animals with normal transmission. Conversely, animals with increased cholinergic signaling paralyze more quickly than animals with normal transmission.

amount of signaling from GABAergic motor neurons (Miller et al., 1996a; Nguyen et al., 1995; Sieburth et al., 2005; Vashlishan et al., 2008), (Fig. 4-1 B). Genetic manipulations that decrease acetylcholine release or AChR function result in resistance to aldicarb (Miller et al., 1996a; Nguyen et al., 1995; Richmond and Jorgensen, 1999; Sieburth et al., 2005). Conversely, animals that have enhanced ACh release or receptor function are hypersensitive to aldicarb's effects. Animals with reduced GABAergic inputs to muscle will also have a hypersensitive phenotype (Vashlishan et al., 2008). For example, mutations in presynaptic calcium channels (*unc-2*), SNARE-binding proteins that promote vesicle priming (*unc-13*), or the kinesin motor responsible for transport of synaptic vesicles (SVs) to synapses (*unc-104*), cause aldicarb resistant phenotypes (Miller et al., 1996a; Nguyen et al., 1995).

To discriminate between changes in presynaptic release versus changes in AChR clustering or sensitivity, aldicarb-induced paralysis can be compared to paralysis induced by AChR agonists, such as levamisol and nicotine. These agonists will hyper-contract muscle and cause rigid paralysis in a similar manner to aldicarb, however because they directly stimulate muscle cells, changes in presynaptic release will not affect levamisol or nicotine-induced paralysis. Levamisol is an agonist for subset of AChRs composed of UNC-29, UNC-38, and UNC-63 subunits (Lewis et al., 1980; Richmond and Jorgensen, 1999). *C. elegans* muscle cells also express AChR subunits called ACR-16 that are not sensitive to levamisol, but are instead activated by nicotine (Touroutine et al., 2005). By assaying changes in levamisol- and nicotine-induced paralysis, we can measure changes in the expression of these different subtypes of AChRs.

Screening animals for changes in sensitivity to aldicarb has led to the identification of multiple novel genes involved in the regulation of synaptic transmission (Miller et al., 1996b; Nguyen et al., 1995; Sieburth et al., 2005), many of which have conserved roles in

mammals. Aldicarb assays have also been used to identify and characterize the G protein-coupled receptor signaling pathways that modulate presynaptic vesicle release (Lackner et al., 1999; Nurrish et al., 1999).

In this chapter, we use the model organism *C. elegans* to study the role of CDK-5 in regulation of synaptic transmission. We find that CDK-5 positively regulates acetylcholine release from the NMJ. CDK-5's kinase activity is required for this increase in release, but the activator CDKA-1/p35 is not absolutely required. Finally, we investigate potential a role for CDK-5 in the regulation of synaptic vesicle priming.

Results

CDK-5 is a positive regulator of ACh release and AChR signaling at the NMJ

To probe for changes in neurotransmitter signaling at the NMJ, we assayed *cdk-5(gm336)* and *cdk-5(ok626)* mutant animals for changes in sensitivity to the inhibitor of acetylcholinesterase aldicarb. These alleles both contain deletions that eliminate the *cdk-5* start codon and are predicted to be nulls (Juo et al., 2007). Both *cdk-5(gm336) and cdk-5(ok626)* mutants were strongly resistant to the paralyzing effects of aldicarb compared to wild type animals (Fig. 4-2 A). Similarly, null mutants of CDK-5's activator, *cdka-1/p35*, were also resistant to aldicarb (Fig. 4-2 B). Interestingly, *cdka-1/p35* mutants were not as resistant as *cdk-5* mutants, possibly indicating that CDK-5 has activators other than CDKA-1/p35.

The decrease in aldicarb sensitivity observed in *cdk-5* and *p35* mutants could be explained either by a loss of ACh release, or a decrease in AChR function in the muscle. To test these alternative hypotheses, we performed a cell-type-specific rescue experiment. We expressed wild-type *cdk-5* cDNA under the control of the *unc-17*/vesicular ACh transporter promoter to restrict *cdk-5* expression to cholinergic neurons only. Expression of *cdk-5* in

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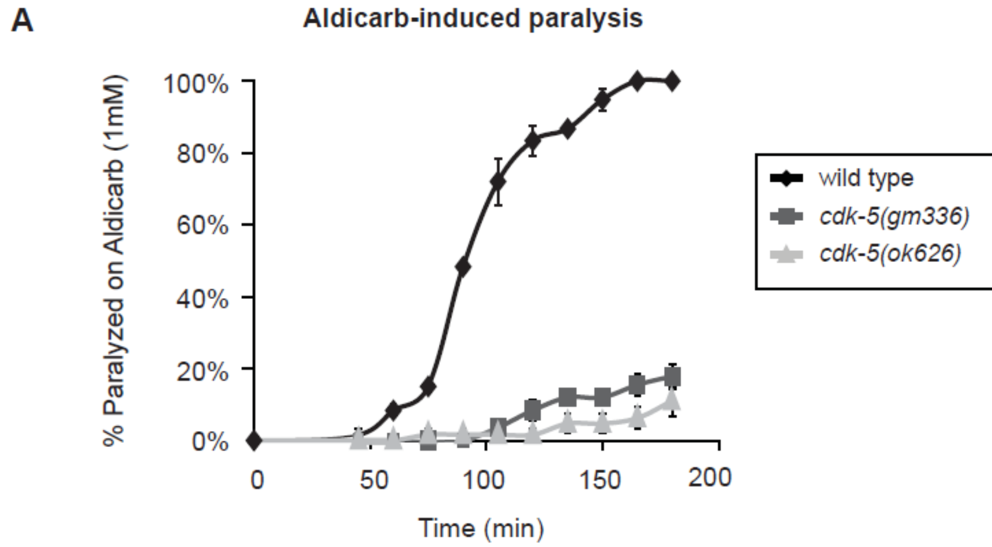
FIGURE 2



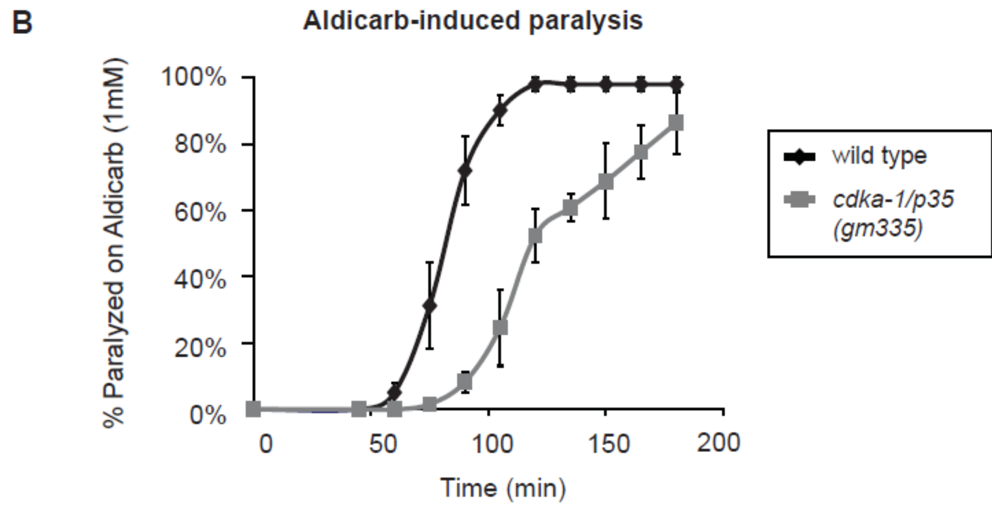


Figure 4-2: *cdk-5* and *p35* mutants have reduced sensitivity to aldicarb (**A**) Aldicarb-induced paralysis in wild type (♦), *cdk-5 (gm336)* (■), and *cdk-5(ok626)* (▲) mutant animals over 3 hours. (**B**) Aldicarb-induced paralysis of wild type (♦) and *cdka-1/p35(gm335)* (■) mutant animals over 3 hours. Paralysis is reported as the percentage of animals that fail to respond to nose touch on a plate of 20 worms. N=3 plates were assayed per genotype.

these neurons was sufficient to fully rescue the reduced sensitivity to aldicarb observed in *cdk-5* mutants, suggesting CDK-5 acts within motor neurons to regulate ACh release (Fig. 4-3 A). This result also verified that changes in aldicarb sensitivity are due to *cdk-5* mutation and not caused by secondary mutations. In addition, overexpression of *Punc-17::cdk-5* in wild-type animals caused them to become hypersensitive to the effects of aldicarb (Fig. 4-3 B). Taken together, these results suggest that CDK-5 acts within cholinergic neurons to positively regulate ACh release.

To test the possibility that some of the decrease in aldicarb sensitivity observed in *cdk-5* mutants was due to changes in postsynaptic receptor expression or function, we exposed *cdk-5* mutants to the AChR agonist levamisol. *Cdk-5(gm336)* and *cdk-5(ok626)* animals showed moderately decreased levamisol sensitivity; however the resistance to levamisol was not as strong as the resistance to aldicarb (Fig. 4-4 A). As a positive control, animals with mutations in the levamisol-sensitive AChR subunit *unc-29* showed no paralysis after 3 hours of exposure to levamisol (Fig. 4-4 A). These findings suggest that CDK-5 may also regulate AChR function in muscle and that this role may partially contribute to aldicarb resistance of *cdk-5* mutants. To verify that changes in levamisol sensitivity were due to *cdk-5* mutation (and not other secondary mutations) and to verify that CDK-5 acts within muscle to regulate levamisol sensitivity, we performed muscle-specific rescue experiments. Wild type *cdk-5* cDNA was expressed under the control of the muscle-specific myosin *myo-3* promoter in *cdk-5(gm36)* mutants. Muscle-specific expression of *cdk-5* not only abolished levamisol resistance, but in fact made *cdk-5* mutants hypersensitive to levamisol (Fig. 4-4 B). This is most likely because expression of the rescue construct exceeded physiological *cdk-5* expression levels. These findings indicate that CDK-5 can act within muscle to act as a positive regulator of either AChR synaptic abundance or AChR sensitivity.

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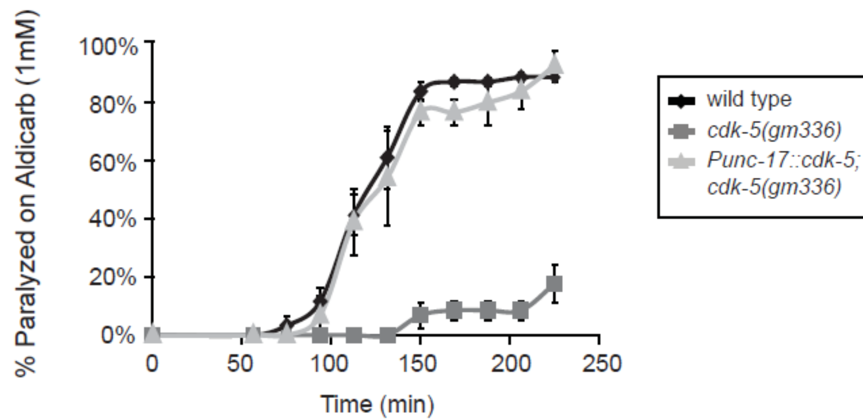
FIGURE 3



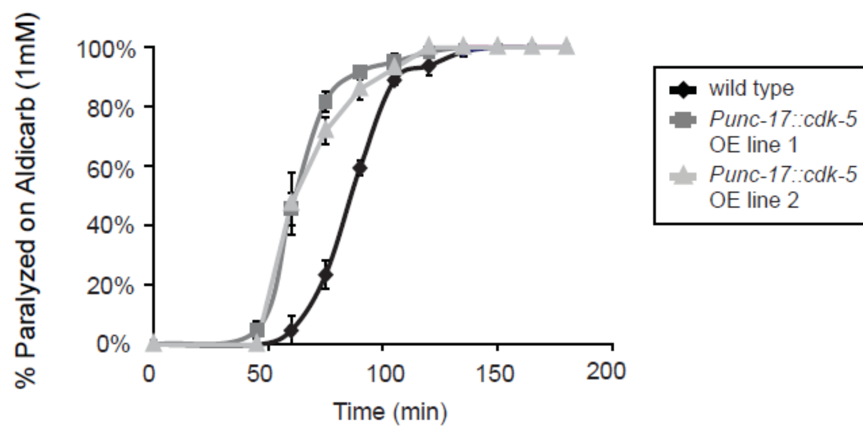


Figure 4-3: *cdk-5* promotes acetylcholine release from neurons (**A**) Aldicarb-induced paralysis in wild type animals (♦), *cdk-5(gm33*6) mutants (■), and *cdk-5 (gm336)* mutants with cholinergic neuron-specific rescue by expression of wild type *cdk-5* cDNA under the control of the *unc-17* promoter (▲). (**B**) Aldicarb-induced paralysis in wild type animals (♦) and animals overexpressing *cdk-5* in cholinergic neurons: *Punc17::cdk-5* OE line 1 (■),*Punc17::cdk-5* OE line 2 (▲). Paralysis is reported as the percentage of animals paralyzed on a plate of 20 worms. N=3 plates were assayed per genotype.

FIGURE 4

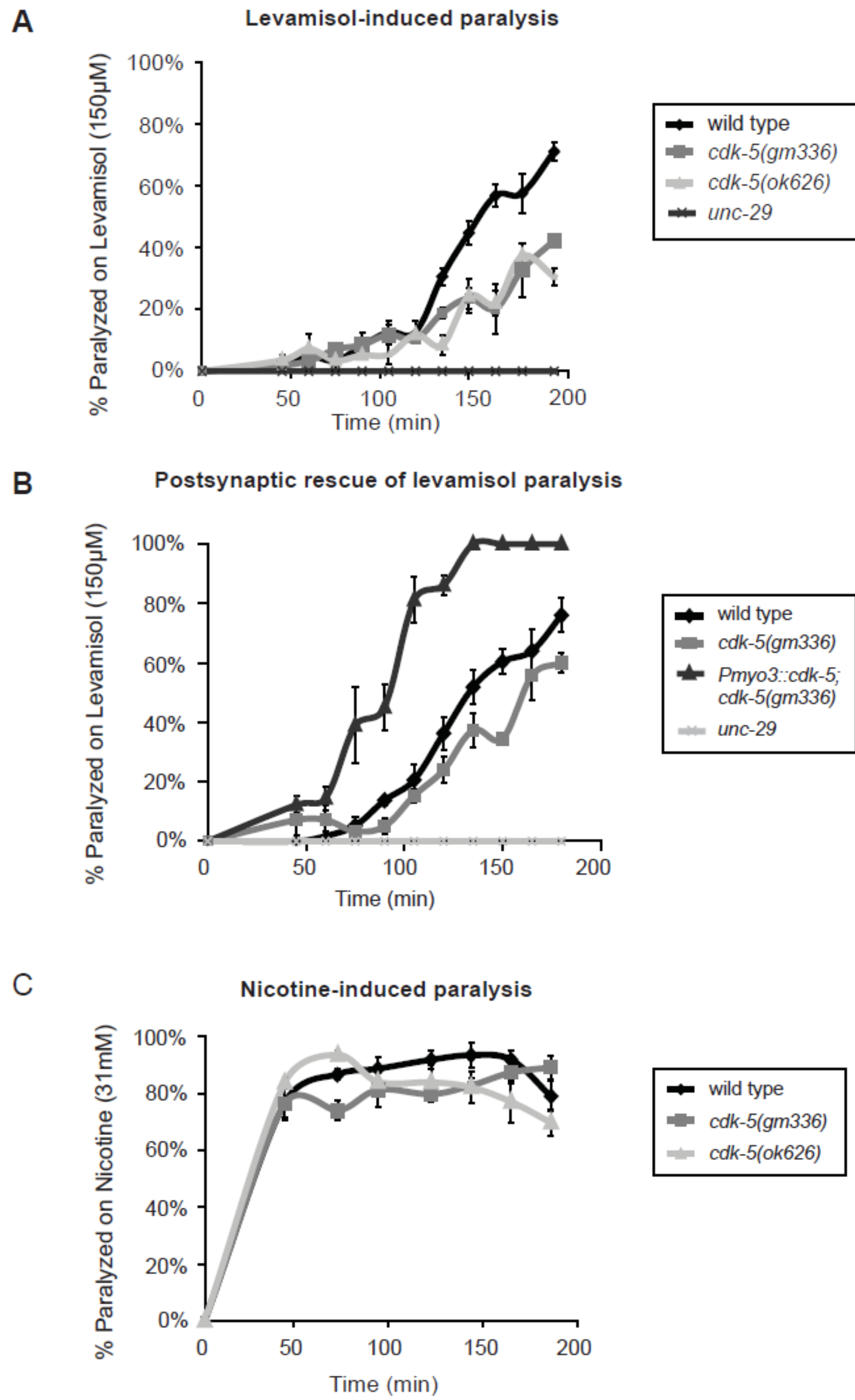


Figure 4-4: Postsynaptic regulation of acetylcholine receptors by CDK-5 (**A**) Levamisol-induced paralysis in wild-type (♦), *cdk-5(gm336)* (■), *cdk-5(ok626)* (▲), and *unc-29(x29)*(**x**) mutant animals over 3 hours. (B) Levamisol-induced paralysis in wild type (♦), *cdk-5(gm336)* mutant animals (■), and *cdk-5(gm336)* mutants with postsynaptic rescue by expression of wild-type *cdk-5* cDNA under the control of the muscle-specific *myo-3* promoter (▲). (C) Nicotine-induced paralysis of wild type (♦), *cdk-5(gm336)* mutant (■), and *cdk-5(ok626)* (▲) mutant animals. Paralysis is reported as the percentage of animals paralyzed on a plate of 20 worms. N=3 plates were assayed per genotype.

Next, we tested for changes in the ACR-16 receptors using a nicotine paralysis assay. *cdk-5(gm336)* and *cdk-5(ok626)* animals show no changes in nicotine-induced paralysis (Fig. 4-4 C), suggesting that CDK-5 may specifically regulate the expression or function of levamisol-sensitive receptors, but not ACR-16 receptors.

In summary, our findings suggest that CDK-5 regulates cholinergic transmission on both sides of the NMJ. However, given that changes in aldicarb sensitivity are much greater than changes in levamisol sensitivity, and that decreased aldicarb sensitivity can be completely reversed by expression of *cdk-5* in cholinergic neurons, it appears that CDK-5's main role at the NMJ is to regulate release of acetylcholine from the presynaptic neuron.

Regulation of CDK-5 kinase activity in NMJ signaling

CDK-5 may potentially regulate ACh signaling via phosphorylation of target proteins or via kinase-independent protein-protein interactions. To test if CDK-5's kinase activity is necessary for its regulation of ACh release, we repeated *cdk-5* overexpression experiments using a kinase-dead version of *cdk-5*. In contrast to overexpression of wild-type *cdk-5*, which caused hypersensitivity to aldicarb, overexpression of kinase-dead *cdk-5* slightly decreased aldicarb sensitivity (Fig. 4-5 A). This indicates that CDK-5's kinase activity is necessary for CDK-5 to enhance ACh release. In fact, the slight reduction in aldicarb sensitivity observed suggests that kinase-dead CDK-5 could potentially act as a dominant-negative.

CDK-5 requires its activator CDKA-1/p35 in order to promote trafficking of GLR-1 receptors to synaptic sites (Juo et al., 2007). We tested if CDKA-1/p35 is required for CDK-5's regulation of synaptic transmission by overexpressing CDK-5 in a *cdka-1/p35* null background. If CDKA-1 is absolutely required for CDK-5 activity, there should be no effect of CDK-5 overexpression in a *cdka-1/p35* null. However, we observed that *Punc-17::cdk-*

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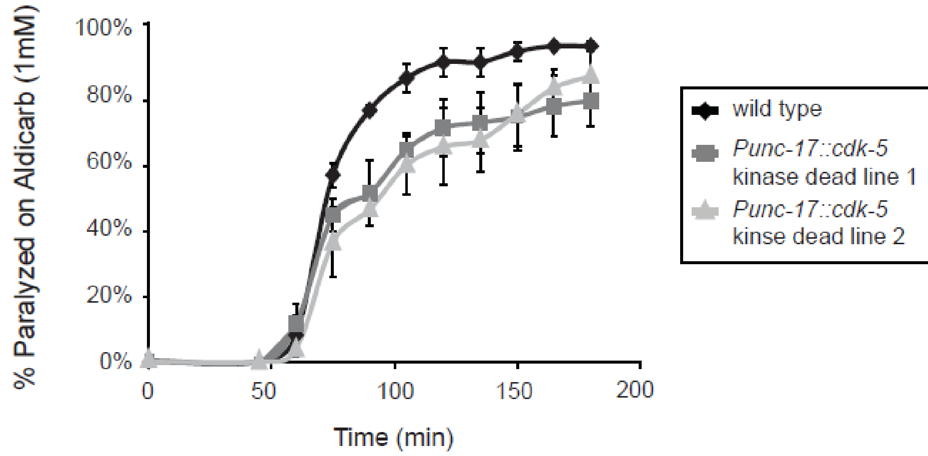
FIGURE 5



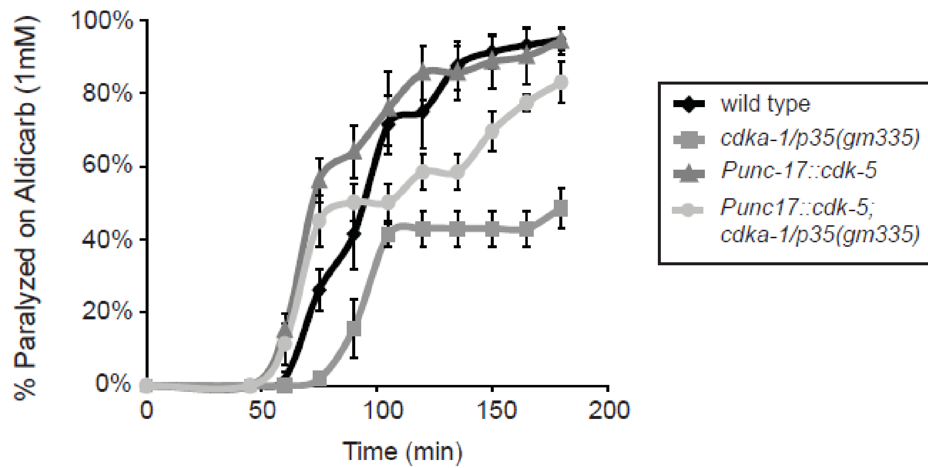


Figure 4-5: CDK-5 kinase activity is required, but p35 is not required for CDK-5-dependent increases in synaptic transmission (**A**) Aldicarb-induced paralysis in wild-type animals (♦), and animal overexpressing kinase-dead *cdk-5* cDNA in cholinergic neurons [*Punc17::cdk-5 kinase dead* line 1 (■); *Punc17::cdk-5 kinase-dead* line 2 (▲)]. (**B**) Aldicarb-induced paralysis in wild-type animals (♦), *cdka-1/p35(gm335)* mutants (■), animals overexpressing *cdk-5* in cholinergic neurons [*Punc17::cdk-5*(▲)], and *cdka-1/p35(gm335)* mutants overexpressing *cdk-5* [*Punc-17::cdk-5; cdka-1/p35(gm335)*(●)]. Paralysis is reported as the percentage of animals paralyzed on a plate of 20 worms. N=3 plates were assayed per genotype.

5;cdka-1/p35(gm335) animals were not as resistant to aldicarb as *cdka-1/p35(gm335)* alone (Fig. 4-5 B). This suggests that CDK-5 can increase synaptic transmission through a CDKA-1/p35-independent pathway.

CDK-5 can stimulate synaptic vesicle release independently of UNC-10

CDK-5 has been shown to phosphorylate many proteins involved in various steps of vesicle release, including synapse formation, SNARE protein binding, and endocytosis (Angelo et al., 2006). In Chapter 2 (Fig. 2-S2), we showed that the amount of RAB-3::GFP at synapses in *cdk-5* mutants is largely unaltered. There is no change in the fluorescence and only a 10% decrease in the density of RAB-3::GFP puncta in *cdk-5* mutants relative to wild type animals (Chapter 2, Fig. 2-S2). This suggested that synapse formation is largely normal in *cdk-5* mutants and that the resistance to aldicarb sensitivity in *cdk-5* mutants is unlikely to be caused by changes in synapse formation or reduced trafficking of synaptic vesicles.

Many genetic mutants which have decreased ACh release and are resistant to aldicarb also have increased fluorescence of GFP-tagged synaptic vesicle proteins at presynaptic sites, due to synaptic vesicle accumulation (Ch'ng et al., 2008; Sieburth et al., 2005). To verify our previous finding that synaptic vesicle abundance at presynaptic sites is not changed in *cdk-5* mutants (Chapter 2 Fig. 2-S2), we also performed quantitative fluorescence imaging using a different synaptic vesicle marker, SNB-1/Synaptobrevin::GFP. Similar to the RAB-3::Venus results, SNB-1::GFP puncta fluorescence and density was not significantly altered in *cdk-5* mutant animals compared to wild type (Figure 4-6 A-D). This confirmed that synapse formation and vesicle trafficking are not altered in *cdk-5* mutants, but did not support the hypothesis that synaptic vesicles accumulate in *cdk-5* mutants.

Mutations in many of the genes involved in synaptic vesicle priming, including *unc-10/Rim-1, egl-8/phospholipase C, tom-1/tomosyn,* and *dgk-1/diacyl glycerol kinase* can alter

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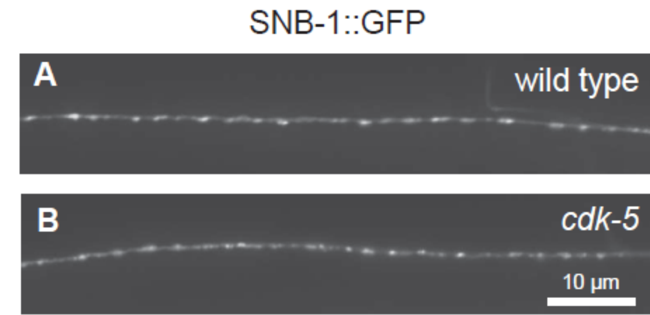
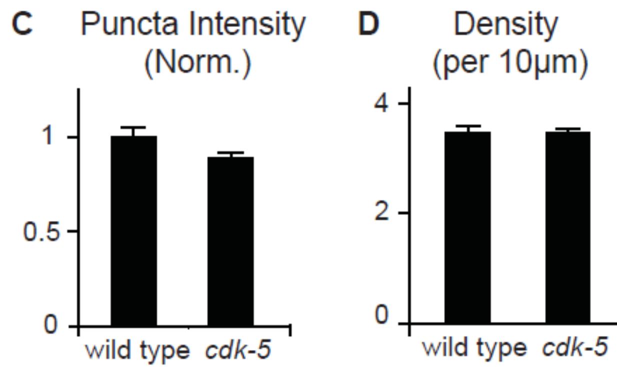
FIGURE 6





E Presynaptic overexpression of *cdk-5* in *unc-10* mutants

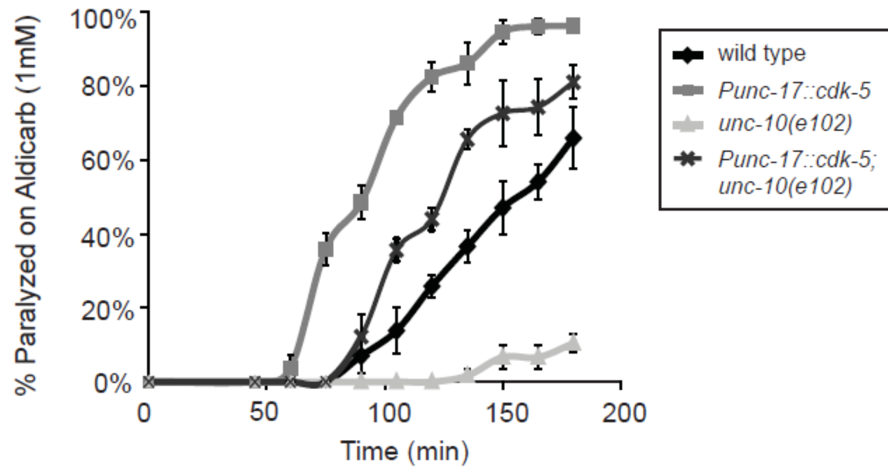


Figure 4-6: *cdk-5* is not required for synapse formation and rescues aldicarb resistance in *unc-10* mutants (**A-B**) Representative images of SNB-1::GFP in the DNC of wild type (**A**) and *cdk-5(gm336)* (**B**). (**C-D**) Quantitative fluorescence analysis of SNB-1::GFP puncta intensity (**C**) and density (**D**) in wild type (n=20) and *cdk-5*(n=24) animals. (**E**) Aldicarb-induced paralysis of wild type animals(♦), *unc-10(e102)* (■), animals overexpressing *cdk-5* in cholinergic neurons [*Punc-17::cdk-5* (▲)], and *unc-10(e102)* mutants overexpressing *cdk-5* [*Punc17::cdk-5; unc-10(e102)*(**x**)]. Paralysis is reported as the percentage of animals paralyzed on a plate of 20 worms. N=3 plates were assayed per genotype.

aldicarb sensitivity without causing significant alterations in the fluorescence of GFP-tagged synaptic vesicle markers at presynaptic sites (Ch'ng et al., 2008; Gracheva et al., 2006; Koushika et al., 2001; Lackner et al., 1999; Nurrish et al., 1999). Thus, we wondered whether CDK-5 might regulate the priming step of vesicle exocytosis. UNC-10/Rim-1 is a Rab-3-binding protein that is believed to promote vesicle priming. *Unc-10* mutants have normal synaptic vesicle docking, but decreased numbers of fusion-competent vesicles at the NMJ (Koushika et al., 2001). These defects can be rescued with an open form of syntaxin, suggesting *unc-10* is required for vesicle priming (Koushika et al., 2001).

We crossed animals overexpressing *cdk-5* in cholinergic neurons with *unc-10* mutant animals to determine if priming by UNC-10 is required for CDK-5's effects on synaptic transmission. *Unc-10(e102)* mutants are strongly resistant to the paralyzing effects of aldicarb (Fig. 4-6 E). However, *cdk-5* overexpression rescued the aldicarb resistance and made *unc-10* mutants moderately hypersensitive to aldicarb (Fig. 4-6 E). This finding suggests that CDK-5 activity may bypass the requirement of UNC-10 in priming of vesicle release.

Discussion

Our data support a role for CDK-5 as a positive regulator of synaptic transmission. We observed that two independent null alleles of *cdk-5* have strongly reduced sensitivity to the AChE inhibitor aldicarb (Fig. 4-2), suggesting CDK-5 promotes synaptic transmission at the NMJ. The decrease in aldicarb sensitivity we observe can be attributed to changes in both cholinergic neurons and body wall muscle. In addition to reduced aldicarb sensitivity, *cdk-5* mutants also have reduced sensitivity to the AChR agonist levamisol, suggesting they have fewer or less functional postsynaptic AChRs (Fig. 4-4). However, because *cdk-5* mutants have only a modest decrease in sensitivity to AChR agonists, compared to a large change in sensitivity to aldicarb, it appears that most of the defect is not due to changes in AChR in the muscle. Although we cannot yet exclude the possibility that *cdk-5* mutants may also be resistant to aldicarb in part because of increased GABAergic signaling, we were able to identify a clear role for CDK-5 in regulation of ACh release from cholinergic neurons. The decreased cholinergic transmission of *cdk-5* mutants can be fully rescued by expression of wild-type *cdk-5* in cholinergic neurons (Fig 4-3). Additionally, overexpression of wild type *cdk-5,* but not kinase-dead *cdk-5,* in cholinergic neurons was sufficient to increase sensitivity to aldicarb in wild type animals, which demonstrates that CDK-5 activity within cholinergic neurons increases ACh release.

In this study, we also found evidence for regulation of CDK-5 by an activator other than CDKA-1/p35. In mammals, *p35;p39* double knock-out animals have the same phenotypes as *cdk-5* knock-outs, implying that these two proteins are the only CDK-5 activators in mammals. No *C. elegans* p39 homolog is predicted in the C. elegans genome, suggesting that *cdka-1/p35* is the sole activator of CDK-5 in worms. Interestingly, although we did observe a decrease in synaptic transmission in animals with mutations in *cdka-1/p35* compared to wild-type animals, the decrease was not as severe as that seen in *cdk-5* null

animals (Fig. 4-2). To test if CDKA-1/p35 is necessary for CDK-5 activity, we overexpressed *cdk-5* in a *cdka-1/p35* null mutant background. These animals had more synaptic transmission (greater aldicarb sensitivity) than *cdka-1/p35* mutants that did not overexpress *cdk-5*, indicating there may indeed be another activator (Fig. 4-5). A recent study by Ou et al (2010) showed that CDK-5 may also be activated by another cyclin-like molecule called CYY-1. It will be interesting to examine if CYY-1 can also activate CDK-5 to regulate synaptic transmission.

In mammals, *cdk-5* knock-out animals have increased clustering of AChRs at the NMJ (Fu et al., 2005). During development, CDK-5 is necessary for ACh-induced dispersal of non-synaptic AChR clusters (Yang, Dominguez et al. 2011). We found that CDK-5 does not play an inhibitory role in AChR clustering in *C. elegans*, but instead, CDK-5 positively regulates AChR abundance or function at the NMJ. Because we saw decreased sensitivity to levamisol but not to nicotine, the receptors affected most likely contain UNC-29, UNC-38, UNC-63, LEV-1, or LEV-8 subunits rather than the ACR-16 subunit (Rand, 2007). Decreased sensitivity to levamisol can be rescued by expression of wild-type *cdk-5* under a muscle-specific promoter, which indicates that CDK-5 acts within muscle itself to regulate receptor function or abundance at the synapse.

Although we found evidence for a postsynaptic role of CDK-5 in regulation of AChR function, we also saw a strong presynaptic effect of CDK-5, suggesting CDK-5 activity may promote vesicle release. This finding is consistent with some previous findings, yet differs from many others. In pancreatic beta cells, CDK-5 has been shown to positively regulate vesicle release at low glucose levels through phosphorylation of Munc-18 (Lilja et al., 2004; Lilja et al., 2001). If, however, insulin release is measured in response to high glucose, CDK-5 appears to act as a negative regulator of release (Wei et al., 2005). CDK-5 has been proposed to negatively regulate release by phosphorylating P/Q calcium channels to reduce

their conductance (Tomizawa et al., 2002), by phosphorylation of SEPT5 to inhibit SEPT5 binding to syntaxin (Amin et al., 2008), and through negative regulation of the size of the releasable pool of vesicles (Kim and Ryan, 2010). Interestingly, at the *Drosophila* NMJ, *cdk-5* mutants have locomotion defects and aberrant growth of motor neuron axon branches, but no change in miniature or evoked excitatory postsynaptic current frequency or amplitude (Kissler et al., 2009).

In this study, we found that overexpression of *cdk-5* rescues the defects in synaptic transmission in *unc-10* mutants. *Unc-10* mutations can be rescued by expression of a constitutively open form of syntaxin (Koushika et al., 2001), raising the possibility that CDK-5 activity could promote priming or syntaxin opening. Ultrastructural and electophysiological studies will be necessary to confirm that CDK-5 regulates synaptic vesicle priming. UNC-18 is a SNARE-binding protein that is critical for vesicle release that has been shown to be phosphorylated by CDK-5 (Li and Chin, 2003). UNC-18 binds to the closed form of syntaxin, and has been theorized both to inhibit priming by keeping syntaxin in its closed form and to act as a chaperone that helps transition syntaxin to its open form to promote priming (Li and Chin, 2003). CDK-5 could potentially phosphorylate UNC-18 to promote syntaxin opening and bypass the need for UNC-10.

Alternatively, phosphorylation of a novel CDK-5 substrate could participate in synaptic transmission in *C. elegans*. Many different proteins make up the exocytic machinery at presynaptic sites, including SNARE proteins (syntaxin, SNAP25, and synaptobrevin), SNARE binding proteins (UNC-13, UNC-18)(Lackner et al., 1999; Nurrish et al., 1999), Rabs (Rab3) and Rab-binding proteins (UNC-10/Rim-1)(Li and Chin, 2003). UNC-13, like UNC-10, promotes vesicle priming and *unc-13* mutations can be rescued by expression of open syntaxin (Richmond et al., 2001). In *C. elegans*, multiple G protein-coupled receptor pathways converge on the priming step of synaptic vesicle exocytosis and

regulate synaptic transmission by modifying UNC-13 function. Activation of the EGL-30/$G_{\alpha}q$ and EGL-8/PLCβ pathway recruits UNC-13 to synapses and enhances synaptic transmission (Lackner et al., 1999). Conversely, activation of GOA-1/$G_{\alpha}o$ decreases the abundance of UNC-13 to inhibit synaptic transmission (Nurrish et al., 1999). Therefore, molecules within these pathways could also be subject to regulation by CDK-5.

Our findings indicate that the *C. elegans* NMJ is a potentially promising model to study CDK-5's regulation of cholinergic synaptic transmission. In both flies and mammals, motor neuron axonal terminals exhibit aberrant branching in *cdk-5* nulls (Fu et al., 2005; Kissler et al., 2009), however we found that the morphology and number of synapses appears to be normal at the NMJ in *C. elegans cdk-5* null mutants (Chapter 2 S2, Fig 4-4). This makes it possible to study CDK-5's role in synaptic transmission without changes in synapse formation confounding the results. Moreover, we were able to show bi-directional regulation of synaptic transmission by CDK-5; loss of function of *cdk-5* reduces synaptic transmission while *cdk-5* overexpression increases synaptic transmission. *cdk-5* expression can therefore be manipulated in both directions to test for interactions with other genes. These findings provide the groundwork for the use of the *C. elegans* NMJ as a model system to screen for novel substrates and regulators of CDK-5.

Materials and Methods

Strains

The following strains were used in this study. N2 bristol, *cdk-5(gm336), cdk-5(ok626), cdka-1/p35(gm335), unc-10(e102), unc-29*(x29*), pzEx6[Punc-17::cdk-5], nuIs152[Punc129::SNB-1::GFP]*, *pzIs6[Punc17::cdk-5]*, *pzIs5* [*Punc17::cdk-5*], *pzEx55[Punc17::cdk-5D133N], pzEx57[Punc17::cdk-5D133N], pzEx78[myo3::cdk-5], nuIs152[Punc129::SNB-1::GFP].*

Paralysis Assays

Aldicarb (Sigma) was dissolved into agar plates at a final concentration of 1mM. For levamisol assays, a final concentration of 150 µm was used. For nicotine assays, final concentration was 31mM. Plates were allowed to cool and spotted with OP50 *E coli*. After overnight incubation of aldicarb plates at room temperature, 20 animals were transferred onto aldicarb plates. After 45 minutes, animals were assayed for paralysis by prodding with a platinum wire. Animals were defined as paralyzed if they failed to respond to prodding with a tail movement. Worms were assayed at 15 minute intervals for 3 hours after first exposure to aldicarb. Experiments were performed in triplicate with the experimenter blinded to the genotype of the animals on each plate.

Imaging

Imaging was performed as described in Chapter 2.

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CHAPTER 5

Discussion

The continuous polarized trafficking of pre- and post-synaptic proteins is essential for neuronal function and survival. The importance of trafficking is highlighted by the fact that mutations in molecular motors and their regulators are linked to neurodegenerative disorders, and that disruption of transport is often an early event in neurodegenerative disease pathogenesis (De Vos et al., 2008). While much is known about the mechanisms that control the trafficking of membrane proteins (Hirokawa et al., 2010), comparatively little is known about how neurons regulate the trafficking of peptide cargoes within dense-core vesicles (DCVs). DCVs can transport neuropeptides and neurotrophins to either the axon terminal or the dendrites and subsequently release these cargoes in an activity-dependent manner (Bergquist and Ludwig, 2008; Landry et al., 2003; Matsuda et al., 2009; Vila-Porcile et al., 2009). Because release of neuropeptides from the two different subcellular compartments occurs in response to different stimuli and can activate different down-stream targets (Bergquist and Ludwig, 2008; Matsuda et al., 2009), the balance of axonal versus dendritic DCVs could potentially impact neuropeptide-regulated behaviors. Moreover, because DCVs also contain neurotrophins (Mowla et al., 1999), the proper trafficking of DCVs may be critical for neuronal survival. Thus, the question of how DCVs are directed towards their ultimate destination is an important question that remains unanswered.

In this thesis, I present research that identifies and characterizes a role for cyclin-dependent kinase-5 (CDK-5) in the regulation of polarized trafficking of DCVs. We find that CDK-5 acts at the *C. elegans* motor neuron cell body to inhibit the dendritic trafficking of DCVs that are destined for the axon (Chapter 2). In addition to this inhibition of dendritic trafficking, we also find that CDK-5 promotes the axonal localization of DCVs, but, interestingly, this role appears to be cell-type specific and separable from its role in negative regulation of DCV trafficking to the dendrite (Chapter 3). Finally, as CDK-5 has been shown to regulate synaptic transmission, a role which might be important for the pathogenesis of

neurodegenerative disease (Coleman and Yao, 2003), I present data which establish the *C. elegans* NMJ as a tractable tool for the investigation of CDK-5-dependent regulation of synaptic transmission (Chapter 4).

CDK-5 negatively regulates trafficking of DCVs to the dendrite

In Chapter 2, we show that CDK-5 regulates the polarized trafficking of DCVs in motor neurons. We find that *cdk-5* loss-of-function mutants have both decreased DCV markers in the axon and increased DCV markers in the dendrite (Chapter 2, Fig. 2-1). CDK-5 does not appear to be important for establishing neuronal polarity, because multiple pre- and postsynaptic-specific proteins have normal localization in *cdk-5* mutants (Chapter 2, Fig. 2-S2). Loading of neuropeptides into DCVs also appears to be normal in *cdk-5* mutants, because the fluorescence of mobile fluorescently-tagged neuropeptide puncta (which likely represent single DCVs), is not altered in *cdk-5* mutants (Chapter 2 Fig. 2-S3). We find that *cdk-5* mutants have increased anterograde trafficking of DCVs and increased stationary DCVs in the dendrite, which indicates that CDK-5 inhibits anterograde trafficking of DCVs to the dendrite rather than promotes retrograde trafficking out of the dendrite. Furthermore, we find that CDK-5 does not promote polarization by enhancing UNC-104-mediated trafficking of DCVs to the axon, nor by preventing UNC-104 from carrying DCVs to the dendrite (Chapter 2, Fig. 2-5). Instead we find that dynein mutations suppress the increase in dendritic DCVs observed in *cdk-5* mutants, suggesting CDK-5 inhibits dynein-mediated trafficking of DCVs to the dendrite (Chapter 2, Fig. 2-6).

Potential outcomes of changes in DCV polarization

In Chapter 2, we find that in the absence of *cdk-5*, the polarized trafficking of INS-22::Venus-containing DCVs towards the axon is disrupted. Why might it be advantageous

for a neuron to polarize DCV trafficking and what might be the consequences of a failure to polarize DCV trafficking? First, polarization of DCVs to the axon could help conserve resources needed to synthesize new DCVs including membrane proteins, propeptide cargoes, and processing enzymes. If neuropeptides must be released from the axon in order to signal properly, then only allowing axonal transport of DCVs prevents DCVs from being wasted in the dendrite. Second, given that DCV release does not occur at the active zone in *C. elegans* (Hammarlund et al., 2008) and can occur in dendrites in mammals (Bergquist and Ludwig, 2008; Ludwig et al., 2002), trafficking of DCVs to the dendrite might result in release of neuropeptides from dendrites. Changing the site of DCV release could result in activation of inappropriate downstream targets. In mammals, dendritic release occurs under different conditions than axonal release (Bergquist and Ludwig, 2008; Ludwig et al., 2002; Matsuda et al., 2009), so increased dendritic trafficking could also potentially alter the circumstances under which neuropeptides are released.

Although DCV markers have been observed in the dendrites of wild-type *C. elegans*, the possibility of dendritic peptide release has not yet been explored. Because the lumen of a DCV has a low pH while the extracellular fluid is closer to neutral, DCV release could potentially be studied by measuring the fluorescence quenching of a pH-sensitive fluorescent tag attached to a DCV membrane protein. In PC12 cells, Taraska et al (2003) utilized the pH-dependent quenching of GFP-tagged phogrin as a method to measure the duration of DCV fusion. pH-sensitive fluorescent markers (pHluorins) have been utilized to study SV release in *C. elegans* (Dittman and Kaplan, 2006), a technique that could be adapted for the study of DCV release. Not only are the sites of DCV release poorly characterized in *C. elegans*, the stimuli that evoke DCV release are also not well described. Development of a pHluorin marker of DCV release could be used to identify stimuli that alter DCV release rates, and determine if different stimuli control release from axons versus

dendrites. Finally, in order to fully understand the consequences of altering the polarized trafficking of DCVs in *C. elegans*, it will be essential to know where the relevant neuropeptide receptors are expressed. Although multiple neuropeptides have been shown to regulate the strength of synaptic signaling at the NMJ (Jacob and Kaplan, 2003; Sieburth et al., 2005; Vashlishan et al., 2008), for many peptides the identity of their receptors are unknown. Some neuropeptide genes have been shown to be expressed in non-neuronal tissues in *C. elegans* (Li, 2008), suggesting that some neuropeptides act as hormonal signals. A better understanding of whether neuropeptides released at the NMJ participate in synaptic, autocrine, or paracrine signaling is necessary to completely understand the implications of altering the polarity of DCV trafficking in *C. elegans*.

Given that dendritic release of neuropeptides occurs in mammalian neurons, it will be interesting to know whether mammalian DCVs are also subject to regulation by CDK-5. In the hypothalamus, release of DCVs from the dendrite has different effects on neuronal firing patterns and behavior than release of DCVs from the axon (Bergquist and Ludwig, 2008). Magnocellular neurons release vasopressin from their axon termini into the periphery to stimulate water retention in the kidneys. Dendritic release of vasopressin, in contrast, has auto-inhibitory effects on these neurons (Bergquist and Ludwig, 2008). Release of oxytocin from the dendrites of magnocellular neurons is also important for auto-regulation of neuronal firing and is crucial for stimulation of lactation (Lambert et al., 1993). In males and non-lactating females, oxytocin is constitutively released from axons, but, during lactation, dendritic release of oxytocin results in autocrine signaling which converts neuronal activity patterns into a bursting pattern, and this bursting results in milk ejection (Lambert et al., 1993). By altering the ratio of DCV trafficking to axons versus dendrites, CDK-5 could affect these auto-regulatory mechanisms, alter hypothalamic neuron firing, and ultimately modify behavior.

Alternative models of CDK-5-dependent inhibition of dynein function

We find that mutations in dynein can suppress the accumulation of DCVs in the dendrites of *cdk-5* mutants, which indicates that dynein is necessary for the increased trafficking of DCVs to the dendrite in *cdk-5* mutants. The simplest model to explain these results is that dynein itself carries DCVs to the dendrite in the absence of *cdk-5*. If this were the case, CDK-5 could either (1) prevent dynein from binding to DCV cargoes or (2) prevent the entry of dynein into the dendrite. We favor the first hypothesis, because if CDK-5 normally inhibits dynein entry into dendrites, none of the cargoes trafficked to or from dendrites could utilize dynein for transport. Fluorescent tagging of dynein to track its movements in the dendrite and its association with DCVs could potentially discriminate between these two possibilities. If CDK-5 regulates the binding of DCVs to dynein, there would be greater co-localization of dynein::GFP with DCV markers in *cdk-5* mutants, but no change in the amount of dynein in the dendrite. Changes in the amount of dynein associated with DCVs could also be assessed biochemically through co-immunoprecipitation. Conversely, if the second hypothesis is correct (CDK-5 prevents dynein entry into the dendrite), we would predict *cdk-5* mutants to have more fluorescently-tagged dynein in the dendrite. An indirect approach to address this question is to determine if other dendritic cargoes which are unaffected by *cdk-5* mutation, such as UNC-9 or Fibrillin, are trafficked to the dendrite by dynein. If dynein is required to traffic these cargoes, it makes the hypothesis that CDK-5 prevents dynein entry into the dendrite less likely.

If indeed CDK-5 does regulate the binding of dynein to DCV cargoes, it raises an interesting question of how retrograde trafficking of DCVs occurs in the axon. In Chapter 2, we show that CDK-5 can act at the cell body to inhibit the entry of DCVs into the dendrite. CDK-5's inhibition of dynein-mediated trafficking might be beneficial at the cell body because it prevents too much dendritic DCV trafficking, but in the axon high CDK-5 activity

might block necessary retrograde trafficking. Loss of retrograde trafficking in the axon can result in neurodegeneration (Koushika et al., 2004). This model might therefore predict the existence of a mechanism to inhibit CDK-5 activity or de-phosphorylate CDK-5 substrates in the axon to facilitate retrograde transport.

Although a model based on the idea that dynein carries DCVs to the dendrite is the simplest explanation of our data, it is not the only possible model. Dynein not only traffics cargoes along microtubules, it is also responsible for the movement of microtubules themselves. Dynein is required to establish the all plus-end out microtubule orientation in the *Drosophila* axon (Stone et al., 2008). Given that dynein activity promotes the all plus-end out orientation of microtubules in the axon, over-activation of dynein might hypothetically increase the percentage of plus-end out microtubules in the dendrite. If this is the case, and CDK-5 is a key inhibitor of dynein, then *cdk-5* mutants might have more plus-end out microtubules in the dendrite compared to wild-type animals. This could then allow a plus-end directed motor to traffic DCVs to the dendrite. We do not favor this interpretation, based on the fact that the normal plus-end motor that traffics DCVs in *C. elegans*, *unc-104,* is not required for the increase in dendritic DCVs observed in *cdk-5* mutants. However, imaging of EBP-1::GFP in *cdk-5* mutants and *dhc-1* mutants will be necessary to rule out this hypothesis.

By eliminating some of the alternative models listed above, we can begin to narrow the focus of our search for potential CDK-5 phosphorylation targets. We demonstrate that dynein is required for the increase in dendritic DCVs seen in *cdk-5* mutants, but the exact phosphorylation target of CDK-5 is still a mystery. CDK-5 phosphorylates Nudel, which directly binds to dynein and is required for dynein-mediated migration of the nucleus and centrosome during neuronal migration, association of dynein with kinetochores during M phase, and targeting of dynein to microtubule plus-ends (Li et al., 2005; Liang et al., 2007;

Niethammer et al., 2000; Shu et al., 2004). Nudel/NUD-2 was shown to be required for the increase in dendritic synaptic vesicles seen in *cdk-5* mutants by Ou et. al. (2010), and was proposed as a potential CDK-5 target. However, mutation of the putative CDK-5 phosphorylation site in NUD-2 did not reveal that site to be critical for regulation of polarized trafficking (Kang Shen, personal communication). Dynein itself could potentially be a CDK-5 substrate, because phosphoproteomic studies indicate that ^{32}P labeling of dynein light intermediate chain decreases after treatment with a CDK-5 inhibitor (Gillardon et al., 2005) and another cyclin-dependent kinase has been shown to phosphorylate dynein light intermediate chain during the cell cycle (Addinall et al., 2001). Another CDK-5 substrate that has recently been shown to bind dynein and inhibit its function is collapsing response mediator protein-2 (CRMP-2). Although originally identified as a microtubule binding protein, CRMP-2 binds to both Kif5 and to dynein and therefore could play a role in the regulation of vesicle trafficking (Arimura et al., 2009). One more CDK-5 substrate that is potentially relevant to DCV release is huntingtin (htt). Expression of htt promotes bi-directional transport of DCVs and the phosphorylation state of htt alters the ratio of anterograde and retrograde DCV trafficking (Colin et al., 2008; Gauthier et al., 2004). Htt can bind dynein intermediate chain and enhance dynein-mediated movement on microtubules (Caviston and Holzbaur, 2009). CDK-5 can phosphorylate htt, which prevents cleavage of the N-terminus of htt by calpain and caspases (Luo et al., 2005). Whether htt also regulates DCV transport in *C. elegans* and if phosphorylation of htt by CDK-5 or htt N-terminal cleavage affects DCV trafficking remains to be determined.

If CDK-5 does not regulate the association of DCVs with dynein, and instead we find that CDK-5 prevents dynein entry into the dendrite, a microtubule-associated protein (MAP) could potentially be the relevant CDK-5 substrate. CDK-5 phosphorylates many MAPs, including tau, CRMP2, doublecortin, and MAP1B (Angelo et al., 2006; Dhariwala and

Rajadhyaksha, 2008; Dhavan and Tsai, 2001). Phosphorylation of tau could potentially affect dynein-mediated trafficking, because encountering a tau cluster on a microtubule increases the likelihood of dynein reversal (Dixit et al., 2008). However, the axon-specific expression pattern of tau makes it less likely that CDK-5 prevents dynein entry into the dendrite through tau phosphorylation (Dehmelt and Halpain, 2005). CRMP2, doublecortin, and MAP1B regulate microtubule stability and polymerization (Dehmelt and Halpain, 2005), but it is not yet clear if these proteins affect the movement of motors along microtubules.

CDK-5 regulates axonal localization of DCVs in DB neurons

In Chapter 2, we present evidence that CDK-5 negatively regulates dendritic trafficking of DCVs and promotes DCV localization to the axon. Because these two changes are concomitant, it seemed likely that each one was caused by the same underlying mechanism. However, when we investigated the role of CDK-5 in regulation of axonal trafficking further in Chapter 3, our findings did not fit this simple model. In our initial studies of DCV localization in Chapter 2, we combined data from DA and DB motor neurons. However, in light of Ou et. al.'s (2010) finding that SV trafficking is regulated differently in DA neurons and DB neurons, we decided to re-evaluate our data by examining results from DA neurons and DB neurons separately. When we performed this analysis, we also found cell-type specific changes in *cdk-5* mutants. *cdk-5* mutants had increased trafficking of DCVs to dendrites in both DA and DB neurons, but the axonal localization of DCVs was only altered in DB neurons. Thus, in the DA neurons of *cdk-5* mutant animals, increased dendritic localization of DCVs is not accompanied by decreased axonal localization, suggesting loss of DCVs into the dendrite does not necessarily cause the decrease in axonal DCVs.

Consistent with this idea, in Chapter 2 we observed equally strong increases in dendritic INS-22::Venus trafficking in *cdk-5* mutants and *cdka-1/p35* mutants, but *cdka-*

1/p35 mutants had a weaker decrease in axonal INS-22::Venus than *cdk-5* mutants (Chapter 2, Fig. 1). This finding shows that the correlation between changes in DCV localization in the axon and dendrite is poor, and suggests that CDKA-1/p35 is not as critical for axonal localization of DCVs as CDK-5. Furthermore, in Chapter 2 we were able to completely rescue the increase in dendritic INS-22::Venus by expression of *cdk-5* under the control of the *unc-129* promoter, but this was not sufficient for complete rescue of the decrease in axonal INS-22::Venus (Chapter 2, Fig.1 and S1). Together, these findings suggest multiple functions of CDK-5; one for regulation of DCV entry into the dendrite (which strongly depends on CDKA-1/p35) and one for regulation of axonal DCV localization (which is less CDKA-1/p35-dependent). The rescue construct we created was sufficient to restore CDK-5's function in the dendrite, but could not fully rescue the function in the axon.

We also found in Chapter 2 that dynein is required for the increase in dendritic DCVs observed in *cdk-5* mutants. Similarly, Ou et al (2010) showed that *cdk-5;cyy-1* mutants have increased retrograde trafficking of SVs in the DA axon, and this increase can be suppressed by dynein mutation. We did not perform time-lapse analysis of mobile DCVs in *cdk-5;cyy-1* double mutants, but given the other similarities between SV trafficking and DCV trafficking in DA neurons, we would predict that retrograde trafficking of DCVs is also increased in the DA axons of *cdk-5;cyy-1* double mutants.

Interestingly, time-lapse imaging of mobile DCVs in *cdk-5* mutant DB axons suggests that loss of inhibition of dynein does not cause the decrease in axonal DCVs in DB neurons. We observed no increase in retrograde trafficking in the DB6 axon, and instead saw a significant reduction in anterograde trafficking and increased stationary DCVs. One simple model to explain why there are more stationary DCVs and decreased anterograde DCV movement in DB axons of *cdk-5* mutants is that CDK-5 prevents anterograde DCVs from becoming stationary. This hypothesis can be explored further through analysis of the

duration of anterograde movements and percent of anterograde puncta that pause in *cdk-5* mutant DB commissures.

A more comprehensive analysis of DCV movement in the axons of DA and DB neurons could prove valuable in discriminating the differences between CDK-5's role in trafficking DCVs in DA neurons versus DB neurons. However, expression of INS-22::Venus under the control of the *unc-129* promoter places constraints on the depth of time-lapse analysis that can be performed in DA and DB axons. Analysis of DCV movement in the dorsal nerve cord is challenging, because DA and DB axons overlap in large sections of the DNC and clustering of INS-22::Venus at release sites creates a high background of stationary puncta. In Chapter 3, we avoided these constraints by analyzing DCV movement in DA and DB commissures, where DA versus DB cell types can be distinguished and there are no background stationary INS-22::Venus puncta. However, the curved morphology of the commissure prevents accurate analysis of the distance a DCV travels, which precludes the measurement of DCV velocity or run length. A single-cell promoter has been described for DA neurons (Klassen and Shen, 2007; Ou et al., 2010; Poon et al., 2008), but, to our knowledge, there is no published description of a single-cell, DB-specific promoter. The development of such a promoter could allow us to measure directionality, velocity, and run length of axonal DCVs in DB neurons specifically. Alternatively, a photoactivatable or photoconvertible DCV marker could be expressed under the *unc-129* promoter, allowing DCVs to be activated/converted specifically in DA or DB cell bodies. Through these experiments, we could test if *cdk-5* mutants have changes in DCV mobility in the dorsal nerve cord. Specifically, we could explore the hypothesis that CDK-5 promotes anterograde transport of DCVs in the DB axon by testing for changes in anterograde DCV velocity and run length.

Finally, epistasis experiments using *unc-31* DCV release mutants suggested that the decrease in axonal INS-22::Venus observed in *cdk-5* mutants might not be due to changes in trafficking at all (Chapter 3). Blocking DCV release with an *unc-31* mutation eliminated the effect of *cdk-5* on axonal INS-22::Venus, suggesting that *cdk-5* mutants have enhanced neuropeptide release. In support of this idea, we only observed *cdk-5*-dependent changes in cell types that rely on UNC-31 for DCV release. *unc-31* mutation does not cause INS-22::Venus to accumulate in DA axons, and, likewise, *cdk-5* mutation does not affect INS-22::Venus localization in DA axons. A pHlourin-based assay of DCV release (described above) could help to confirm the role of CDK-5 in DCV release. The identification of the CDK-5 substrates that mediate these changes in DCV trafficking and DCV release, combined with mutagenesis of CDK-5's phosphorylation sites on these substrates, will ultimately allow us to test whether decreased trafficking or increased release causes reduced axonal INS-22::Venus in *cdk-5* mutants.

CDK-5 has cell-type specific effects on axonal DCV localization

Our finding that *cdk-5* mutants have reduced DCV localization to axons specifically in DB axons (Chapter 3), combined with studies of CDK-5's role in regulation of SV trafficking (Ou et al., 2010), indicate that CDK-5 has cell-type specific effects. Ou et. al. (2010) discovered that CDK-5 and CYY-1 work in parallel, redundant pathways to promote the localization of SVs to presynaptic sites in DA neurons. Surprisingly, using electron microscopy analysis, they also found that in DB neurons both CDK-5 and CYY-1 are dispensable for trafficking of SVs. Our results concur with this report, in that we find that CDK-5 and CYY-1 act redundantly to promote localization of DCVs to DA axons. However, in disagreement with their findings, we found that *cdk-5* is required to promote localization of DCVs to axons in DB neurons. This shows that while SVs and DCVs use similar

mechanisms to polarize their trafficking in DA neurons, the trafficking mechanisms of SVs and DCVs are different in DB neurons.

This finding was remarkable in light of the fact that DA neurons and DB neurons are seemingly quite similar. Both are cholinergic motor neurons with ventral dendrites and dorsal axons, and both are important for directed locomotion. The two cell types differ in that DAs control backward locomotion and have anteriorly projecting processes, while DBs control forward locomotion and have posteriorly projecting processes (Haspel et al., 2010; White et al., 1986). DA neurons receive synaptic inputs from AVA and AVD/E interneurons, while DBs receive inputs from AVB and PVC interneurons (White, 1986). Both cell types synapse onto muscle and VD inhibitory neurons (Chalfie and White 1988). Despite the appearance of similarity, it seems that these two cell types differ in the molecular mechanisms they utilize to regulate axonal vesicle trafficking. In the future, these two cell types should be investigated separately and mechanisms that regulate protein localization in one cannot always be assumed to be important in the other.

CDK-5 and CYY-1 act in parallel and redundant pathways

Similar to Ou et al (2010), we found that CDK-5 and PCT-1 kinases function in parallel and redundant pathways to promote localization of DCVs to the DA axon (Chapter 3). It is not yet clear if this is due to the fact that the two kinases phosphorylate the same SP or TP site on the same target protein, or if they phosphorylate different proteins which have redundant functions. Ou et al (2010) also found that there is cross-talk between these two pathways, because CYY-1, which is essential for PCT-1 activity, also activates CDK-5. Thus, increased *cyy-1* expression could be both a mechanism for CDK-5 activation and a compensatory mechanism for loss of *cdk-5* function. It will be interesting to see if CDK-5 and CYY-1 act in parallel pathways or the same pathway for regulation of synaptic

transmission at the *C. elegans* NMJ. In mammals, Pctaire kinase can be phosphorylated by CDK-5 at S95 (Cheng et al., 2002), but this site is not conserved in *C. elegans* (Ou et al., 2010). Like CDK-5, Pctaire kinases have been shown to regulate vesicle exocytosis and phosphorylation of tau in mammalian neurons (Herskovits and Davies, 2006; Liu et al., 2006). This raises the question of whether the evolution of a CDK-5 phosphorylation site in Pctaire kinase altered the relationship between these two kinases and whether they act in parallel pathways in nematodes but act in the same pathways in mammals.

CDK-5 negatively regulates neuropeptide release, but positively regulates synaptic vesicle release

In Chapter 3, we show that the decrease in DCV marker fluorescence seen in *cdk-5* mutants is completely rescued by blocking DCV release through mutation of *unc-31*. This suggests that *cdk-5* mutants have enhanced DCV release and that CDK-5 negatively regulates neuropeptide release. While this was consistent with studies of CDK-5's regulation of DCV release from pancreatic beta cells and chromaffin cells (Barclay et al., 2004; Wei et al., 2005), this was in stark contrast to our findings from our studies of CDK-5 function using the aldicarb assay (Chapter 4). We found that CDK-5 positively regulates the amount of cholinergic signaling at the NMJ by acting within cholinergic motor neurons, suggesting CDK-5 promotes acetylcholine release.

This discrepancy is also puzzling in light of the fact that neuropeptide release has generally been shown to positively regulate synaptic transmission at the NMJ. Although individual peptides can have inhibitory effects at the NMJ (Vashlishan et al., 2008), mutations in neuropeptide processing enzyme genes or genes that promote neuropeptide release, including the *egl-3/ proprotein convertase, egl-21/carboxypeptidase E*, *unc-31/CAPS* and *pkc-1*, cause aldicarb resistance (Jacob and Kaplan, 2003; Sieburth et al.,

2005; Sieburth et al., 2007). Mutations in *ins-22* also cause aldicarb resistance (Sieburth et al., 2005), so enhanced neuropeptide release in general and enhanced release of INS-22 specifically would be predicted to increase aldicarb sensitivity.

These conflicting findings might be explained by multiple roles of CDK-5 in different subsets of cholinergic neurons. Our evidence for CDK-5's role in regulation of neuropeptide release comes from imaging of a specific subset of motor neurons, the DB neurons, whereas our experiments that measured synaptic vesicle release using the aldicarb assay are affected by acetylcholine release throughout the whole animal. Our aldicarb experiments examining the effects of overexpression of *cdk-5* used a promoter that induced *cdk-5* expression in all cholinergic neurons, not just DB neurons. If CDK-5 was positive regulator of vesicle release in all the other cholinergic neurons besides DBs, the overall effect might be to increase synaptic transmission at the NMJ rather than decrease it. The development of better DA-specific and DB-specific promoters will be necessary to confirm or reject this hypothesis.

Alternatively, CDK-5 could increase signaling at the NMJ by decreasing the release of an inhibitory peptide. RNAi knock-down of neuropeptide receptors *npr-1*, a neuropeptide Y receptor homolog, and Y54E2A.1, a Galanin receptor homolog causes increased NMJ signaling (Vashlishan et al., 2008) and direct application of some neuropeptides onto pharyngeal muscle can reduce the rate of muscle contraction (Rogers et al., 2001). If CDK-5 inhibits release of inhibitory peptides that have strong effects on aldicarb sensitivity, *cdk-5* mutants might be resistant to aldicarb because of enhanced inhibitory neuropeptide signaling. However, this model seems unlikely to be correct in light of our finding that overexpression of *cdk-5* in cholinergic neurons can rescue defects in synaptic transmission observed in *unc-10* mutants. *Unc-10* mutants are uncoordinated, are robustly resistant to aldicarb, and have a five-fold reduction in miniature postsynaptic current frequency at the

NMJ due to a failure of vesicle priming (Koushika et al., 2001). Because neuropeptides play a modulatory role in synaptic transmission and CDK-5 only inhibits the release of neuropeptides from one subset of motor neurons, it seems unlikely that CDK-5's effects on neuropeptide release alone would be strong enough to compensate for loss of *unc-10*.

Finally, it may be the case that CDK-5 is both a positive regulator of synaptic vesicle release in cholinergic neurons and a negative regulator of DCV release in a subset of these neurons. If CDK-5's role in synaptic vesicle release has a stronger effect on aldicarb sensitivity than its effect on DCV release, the overall phenotype of a *cdk-5* mutant animal would be aldicarb resistance. SVs and DCVs share similar release properties and some of the same release machinery, including RAB-3, RAB-27, and UNC-13 (Gracheva et al., 2006; Hammarlund et al., 2008; Li and Chin, 2003; Madison et al., 2005; Mahoney et al., 2006; Weimer et al., 2006; Yu et al., 2008). On the other hand, there are some proteins that specifically regulate DCV release, including UNC-31/CAPS and PKC (Hammarlund et al., 2008; Sieburth et al., 2007; Speese et al., 2007). If one of these DCV-specific proteins were a CDK-5 substrate, that could explain why CDK-5 appears to be a positive regulator of SV release but a negative regulator of DCV release. SV and DCV also differ in that there is strong evidence to support the idea that DCVs can undergo "kiss-and-run" fusion events, where a fraction of the contents of their lumen are excreted, while kiss-and-run events are have been shown to only represent a small fraction of the SV release events at the NMJ (An and Zenisek, 2004; He and Wu, 2007; Taraska et al., 2003; Verstreken et al., 2002). Inhibition of CDK-5 has been reported to increase quantal size and lengthen the duration of kiss-and-run fusion events in chromaffin cells (Barclay et al., 2004). One possibility to consider is that CDK-5 may shorten the duration of kiss-and-run fusion of DCVs, but has different effects on full-fusion events that occur during synaptic vesicle release.

Concluding remarks

In this thesis, I present data that point to multiple roles for CDK-5 in the regulation of neuronal function in *C. elegans*, including regulation of polarized DCV trafficking, inhibition of neuropeptide release, and positive regulation of acetylcholine release at the NMJ. I focused my studies on the cholinergic motor neurons of *C. elegans*, but even within this small, seemingly homogeneous group of neurons I found different roles for CDK-5 in the different cholinergic motor neuron subtypes. This finding was intriguing due to the fact that these two cell types release the same neurotransmitter onto the same postsynaptic targets (White, 1986), and they have very similar morphologies and perform related functions during locomotion (Haspel et al., 2010; White et al., 1986). These findings reflect the state of the wider field of CDK-5 research; the importance of CDK-5 in neurons has grown rapidly over the years as more substrates have been identified, but analysis of CDK-5's function can become complicated by its multiple effects within one cell (Angelo et al., 2006; Dhariwala and Rajadhyaksha, 2008; Dhavan and Tsai, 2001; Hawasli and Bibb, 2007). This heterogeneity of functions is perhaps not surprising in a kinase, which can phosphorylate multiple substrates to modify their functions. This raises the question of how a neuron can restrict CDK-5 activity so that it only phosphorylates particular target proteins. A greater depth of knowledge of the conditions and mechanisms that regulate CDK-5 activity is needed to fully understand the role of CDK-5 activity in regulation of nervous system function.

In this work, I present evidence that CDK-5 may play opposing roles in the regulation of DCV and SV release. Imaging of INS-22::Venus in *cdk-5* mutant axons suggested a role for CDK-5 as a negative regulator of DCV release, while analysis of *cdk-5* mutant sensitivity to aldicarb suggested that CDK-5 positively regulates SV release. Interestingly, overexpression of *cdk-5* in cholinergic neurons is sufficient to rescue defects in synaptic

transmission in *unc-10* mutant animals, suggesting CDK-5 may act at a post-docking step during synaptic vesicle exocytosis. Whether this is due to CDK-5-dependent phosphorylation of a previously identified substrate of CDK-5, or if it represents a novel site of CDK-5 action is not yet clear. My work provides the basis for future screening of known regulators of synaptic transmission at the NMJ to search for novel CDK-5 substrates and up-stream regulators of CDK-5.

Our finding that *cdk-5* mutants have defects in polarized trafficking of DCVs reveals a novel role for CDK-5 and may reveal new CDK-5 substrates. Although we determined that dynein is required for increased trafficking of DCVs to the dendrite in *cdk-5* mutants, we have not yet identified a direct target of CDK-5. The ease of forward genetic screening and genome-wide RNAi screening in *C. elegans* will make it possible in the future to perform an unbiased search for the relevant CDK-5 target and will also allow the identification of other genes that are required for the polarized trafficking of DCVs. In our studies, we focused on one tagged neuropeptide, INS-22::Venus, and verified our findings by examining tagged DCV membrane protein, IDA-1::GFP. However, DCVs carry diverse neuropeptide cargoes, and the mechanisms that regulate the trafficking of INS-22 and IDA-1 may not regulate all neuropeptide trafficking. It will therefore be interesting to compare our findings across a range of tagged neuropeptides and to search for genes that are involved in the trafficking of specific subsets of DCVs, such as DCVs targeted to dendrites.

Researchers have only just started to scratch the surface of knowledge of neuropeptide function. The number of identified neuropeptides in mammals and predicted peptides in *C. elegans* reaches into the hundreds (Li, 2005; Li and Kim, 2008; Nathoo et al., 2001), and it has become clear that co-transmission of classical neurotransmitters and neuropeptides is the rule and not the exception (Mains and Eipper, 1999). However, the functions and receptors of many neuropeptides are largely unknown. DCVs carry diverse

neuropeptide cargoes, but we currently know little regarding how the trafficking of these heterogeneous cargoes is achieved. Different peptides can localize to different subcellular compartments in mammalian neurons and Aplysia bag-cell neurons (Fisher et al., 1988; Landry et al., 2003), suggesting that different populations of DCVs can engage different trafficking mechanisms. We do not yet understand how this differential trafficking is achieved, or even which molecules mediate the dendritic trafficking of DCVs. Neuronal activity has been shown to regulate DCV trafficking dynamics in mammalian neuronal cultures and at the *Drosophila* NMJ (de Wit et al., 2006; Shakiryanova et al., 2006), but it remains to be determined if the trafficking of all DCV cargoes is regulated by activity, or if different subsets of DCVs respond differently to changes in neuronal activity. Finally, it is not clear if the balance of dendritic versus axonal trafficking of DCVs remains constant over a neuron's lifetime, or if it can be changed in response to different stimuli. Regulation of the axodendritic polarity of DCV trafficking could be crucial for the control of behaviors that are modulated by neuropeptides, and is an almost entirely uncharted area of research.

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