Prospectus

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Netrin-mediated molecular mechanisms of presynaptic assembly

During neural development, individual neurons must navigate developing nervous systems with remarkable precision. Once in the proper location, neurons choose the correct synaptic partners to form functional neural circuits, a process known as synaptic specificity (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). I am interested in understanding how synaptic specificity is regulated at a molecular level.

Netrin is an extracellular molecule implicated in axon guidance, providing neurons with spatial instructions during nervous system development (Tessier-Lavigne, 1996). In *C. elegans*, UNC-6 (Netrin) also mediates the development of synapses between two interneurons (AIY and RIA) through its receptor UNC-40/DCC (Colón-Ramos, 2007). In *unc-40* loss-of-function mutants, the postsynaptic neuron RIA exhibits disrupted guidance, while axon guidance is **not** affected in the presynaptic neuron AIY. Instead, UNC-40/DCC is cell-autonomously required in AIY for proper presynaptic assembly. Moreover, we have found that the cytoplasmic molecule MIG-10/Lamellipodin transduces the Netrin signal in AIY to instruct presynaptic assembly. I propose to understand how Netrin regulates presynaptic assembly in AIY. I hypothesize that upon Netrin binding, the Netrin receptor UNC-40 interacts with members of the Rac pathway to promote presynaptic assembly through MIG-10.

I propose to address the following specific aims:

I: To identify the MIG-10 isoform involved in presynaptic assembly downstream of UNC-40 in AIY. There are three MIG-10 isoforms in *C. elegans*. I propose to determine which isoform directs presynaptic assembly downstream of Netrin. I will use recombinogenic engineering to selectively disrupt the MIG-10 isoforms in rescue experiments to identify isoform requirement for presynaptic assembly. Determining which MIG-10 isoform functions in presynaptic assembly in AIY will inform our understanding of the molecular pathways activated by Netrin in presynaptic assembly.

II: To examine how UNC-40 genetically interacts with MIG-10 during presynaptic assembly. UNC-40/DCC genetically interacts with PI3K and Rac GTPases to transduce its activity during axon guidance (Round and Stein, 2007). We have preliminary evidence that PI3K is not involved in presynaptic assembly, but that RacGTPases are involved in presynaptic assembly. I will first determine which participants in the Rac pathway are required during Netrin-mediated presynaptic assembly through loss-of-function mutants. Furthermore, I will determine whether they mediate the MIG-10:UNC-40 genetic interaction with epistasis and protein localization experiments. These studies will elucidate how Netrin genetically interacts with MIG-10 in directing presynaptic assembly.

III: To identify the molecular pathways activated downstream of MIG-10 that instruct presynaptic assembly in AIY. I hypothesize that the effectors of MIG-10 in the presynaptic assembly module are distinct from MIG-10 effectors identified in the axon guidance module. Talin is a known effector of MIG-10 that is not implicated in axon guidance. Thus, I will analyze *talin* mutants for presynaptic assembly defects similar to *unc-40*. Epistasis studies will help organize these genes into pathways downstream of MIG-10 that instruct presynaptic assembly. In addition, a genetic screen will identify novel effectors of MIG-10. This analysis will be an important component in understanding the novel pathway downstream of MIG-10.

Background and Significance

Synapses are specialized intercellular junctions

Once an axon has reached its correct location, the growth cone machinery must transition from interpreting guidance cues to responding to cues promoting synapse assembly. There are stereotyped steps in synapse formation in both vertebrates and invertebrates. First, the axon growth cone and postsynaptic dendrite must come in contact. Second, specific proteins are recruited to the pre-and postsynaptic assemblies at the developing synapse. Third, young synapses mature or are degraded due to activity levels and the presence or absence of synaptic patterning molecules (Arikkath and Reichardt, 2008; Colon-Ramos, 2009; Gerrow and El-Husseini, 2006; Lardi-Studler and Fritschy, 2007).

Recognition of synaptic targets requires precision and accuracy, as proper connections are necessary for the configuration of functional neural circuits. The specificity, or location, of the initial contact between pre- and postsynaptic neurons is thought to be mediated by cell adhesion molecules, such as cadherins, integrins, neurexins, neuroligins, and ephrins. These molecules interact extracellularly to participate in target recognition, to stabilize the synapses by physically linking the synaptic partners and to differentiate between the pre- and postsynaptic cells (Akins and Biederer, 2006; Arikkath and Reichardt, 2008; Colon-Ramos, 2009; Dalva et al., 2007; Gerrow and El-Husseini, 2006; Lardi-Studler and Fritschy, 2007; Waites et al., 2005). The cytoplasmic tails of cell adhesion molecules contain protein binding domains that link these molecules to the cytoskeleton and permit signal transduction, thus instructing proper synaptic partner recognition (Yamagata et al., 2003).

Classically, the second step in synaptogenesis involves the recruitment of pre- and postsynaptic proteins to the respective sides of the synapse. Specific kinesin-like motor proteins, such as UNC-104, transport synaptic vesicles, containing neurotransmitters, to the periactive zones of presynaptic regions (Jin and Garner, 2008). In the postsynaptic neuron, neurotransmitter receptors accumulate at the synapse (Arikkath and Reichardt, 2008; Gerrow and El-Husseini, 2006). Concurrent with or subsequent to pre- and postsynaptic differentiation, the synapse is stabilized with additional interactions between cell adhesion molecules (Arikkath and Reichardt, 2008; Colon-Ramos, 2009; Gerrow and El-Husseini, 2006). Interestingly, pre- and postsynaptic assemblies have been observed prior to cell contact and recognition, suggesting that molecules other than cell adhesion molecules can specify synapse location and formation (Colon-Ramos, 2009).

Initial synapse formation is not perfect; there is some degree of mismatch between pre- and postsynaptic partners (Akins and Biederer, 2006). During vertebrate embryonic development, neurons form transient synapses (Waites et al., 2005). Activity of the synapse provides cues to strengthen or eliminate the new synapse to further fine-tune the specificity of synaptic connections and modulate the neural circuit (Arikkath and Reichardt, 2008; Gerrow and El-Husseini, 2006; Lardi-Studler and Fritschy, 2007). Thus, this synaptic plasticity indicates that initial synapse formation is not sufficient for synapse survival; other cues are required to maintain synapses.

Neurons navigate the complex embryonic environment during axon guidance

In the human nervous system, each of over 100 billion neurons must navigate the spatiotemporal matrix of the developing embryo to form over 100 trillion synapses. This migration is accomplished with astounding accuracy and precision through the direction of extracellular cues and their receptors (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). These extracellular cues can be

organized into attractive or repulsive instructions, each of which can be further characterized as acting over long or short ranges (Tessier-Lavigne and Goodman, 1996). Netrins, Slits, Ephrins, Semaphorins, Wnts, sonic hedgehog, and bone morphogenic proteins (BMPs) have been implicated as molecular cues for axon guidance in vertebrate and invertebrate systems (Dickson, 2002; Gitai et al., 2003; Quinn and Wadsworth, 2008; Tessier-Lavigne and Goodman, 1996).

A developing neuron sends out an axon with a growth cone at its protruding tip; the growth cone is sensitive to specific guidance cues that allow it to grow in a stereotyped manner to connect with its correct synaptic partners (Tessier-Lavigne and Goodman, 1996). The extracellular cues activate or repress signaling cascades that modulate cytoskeletal dynamics, thus regulating axon growth toward or away from a gradient of the extracellular cue. The direction of axon guidance is determined by asymmetric accumulation of microtubules and actin within the growth cone (Chang et al., 2006; Dickson, 2002; Gitai et al., 2003; Quinn and Wadsworth, 2008).

Netrin's role in directing axon guidance

Netrin, a member of the laminin family, is highly conserved across vertebrates and invertebrates and can function both as an attractive or repulsive axon guidance cue (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). The *C. elegans* Netrin homolog, UNC-6, has two conserved transmembrane receptors, UNC-40/DCC (Deleted in Colorectal Cancer) and UNC-5. UNC-40/DCC alone participates in attraction of growth cones toward a Netrin gradient while UNC-5 has been implicated in growth cone repulsion away from a Netrin gradient (Dickson, 2002; Hong et al., 1999; Round and Stein, 2007).

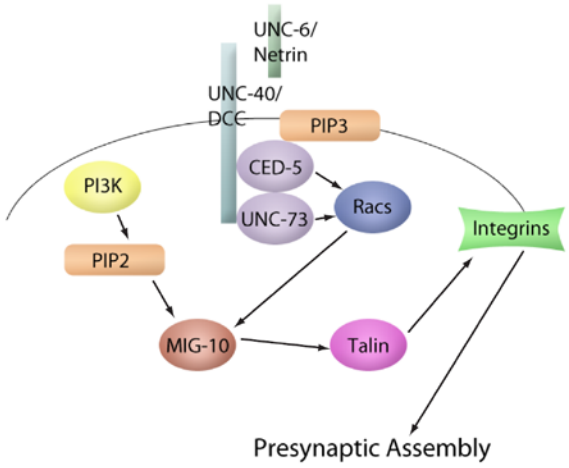

Figure 1. Signaling pathways downstream of UNC-40 involving MIG-10. CED-5 and UNC-73 are *C. elegans* Rac GEFs (Quinn and Wadsworth, 2008). The depicted pathways do not represent all of the pathways identified downstream of Netrin.

The cytoplasmic region of UNC-40/DCC does not contain any catalytic domains; rather, Netrin activation of UNC-40/DCC results in protein effectors binding to its cytoplasmic tail (Round and Stein, 2007). The molecules implicated downstream of UNC-40/DCC can be generally organized into five signaling pathways. One of these modules is the mitogen-activated protein kinase (MAPK) cascade. This cascade is important in influencing second messenger activity that ultimately stimulates local protein synthesis necessary for axon guidance (Forcet et al., 2002; Round and Stein, 2007). Netrin binding also activates a second signaling pathway that results in initiation of NFAT activity. NFAT is a nuclear factor associated with transcription complexes that transcribe genes required for axon migration (Round and Stein, 2007). A third pathway triggered by Netrin involves phospholipase Cγ (PLCγ). PLCγ has been hypothesized to act as a negative regulator of membrane localization of UNC-5 (the repulsive Netrin receptor) through protein kinase C (PKC). This would ensure that the growth cone maintains an attractive response to Netrin (Round and Stein, 2007). A fourth pathway includes activation of Wiskott-Aldrich syndrome protein (WASP), which subsequently modulates actin

cytoskeleton dynamics in the growth cone (Round and Stein, 2007). WASP has been copurified with WAVE, Ena/VASP and Lamellipodin in a complex from mammalian brain. As Ena/VASP also mediates actin dynamics, this pathway broadly regulates the growth cone cytoskeleton (Chang et al., 2006).

Of particular importance to this proposal, phosphatidylinositol 3-kinase (PI3K) and RacGTPases also act downstream of UNC-40/DCC and mediate its genetic interaction with MIG-10 (Adler et al., 2006; Chang et al., 2006; Lundquist et al., 2001; Quinn et al., 2008; Quinn and Wadsworth, 2008; Round and Stein, 2007). AGE-1/PI3K regulates MIG-10/Lamellipodin through the production of phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2 or PIP2), which binds to the PH domain of MIG-10 (Fig. 5A). MIG-10 is important in regulating lamellipodial dynamics and proper axon guidance (Chang et al., 2006; Krause et al., 2004; Round and Stein, 2007). Furthermore, Rac GTPases also mediate axon guidance downstream of UNC-40/DCC (Chang et al., 2006; Lundquist et al., 2001; Quinn et al., 2008). The Rac pathway influences asymmetric distribution of MIG-10 through interaction with the Ras-association (RA) domain of MIG-10 (Chang et al., 2006). I will focus my studies on the Rac pathway, as our preliminary results have indicated that RacGTPases affect presynaptic assembly in AIY (Fig. 1).

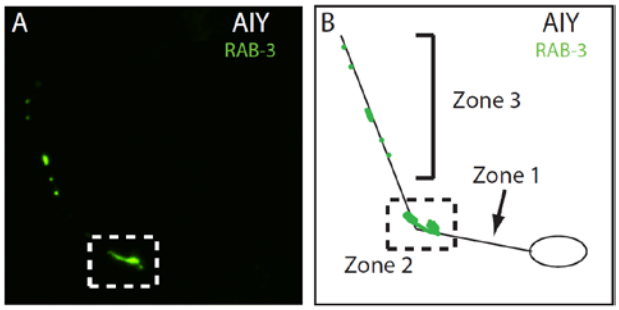

Figure 2. Presynaptic specialization in AIY. (A) Visualization of presynaptic assemblies in AIY in wild type animals. (B) Schematic of A. The AIY interneuron can be divided into three regions, Zone 1 (asynaptic), Zone 2 (synapses with RIA) and Zone 3 (synapses with AIZ). RAB-3:GFP labels synaptic vesicles which localize to presynaptic structures.

Netrin instructs presynaptic assembly in AIY through MIG-10

AIY is an interneuron involved in the thermotaxis circuit in *C. elegans*; it has two main postsynaptic partners, RIA and AIZ (Mori and Ohshima, 1995). AIY synapses with RIA in a region we termed Zone 2 and synapses with AIZ in Zone 3 (Fig. 2) (Colon-Ramos et al., 2007).

We previously identified UNC-40/DCC involvement in presynaptic assembly in AIY with a GFP fusion construct that labels synaptic vesicles specifically in AIY. In wild type animals, this fusion protein consistently labels AIY Zone 2 and Zone 3 in a stereotyped manner while exhibiting little or no signal in the cell body or Zone 1 (Fig. 2). With colocalization studies, this indicates that AIY develops presynaptic assemblies only in Zone 2 and Zone 3. In *unc-40* loss-of-function mutants, RIA axon guidance is disrupted, demonstrating that UNC-40 instructs axon guidance in RIA (Fig. 3, compare B, E to A, D). However, AIY exhibits no guidance defects in *unc-40* or *unc-6* loss-of-function mutants. Our research indicates that Zone 2 is disrupted in *unc-40* or *unc-6* loss-of-function mutants containing the synaptic vesicle marker (Fig. 3H,K). Thus, UNC-6/Netrin, which was discovered and previously studied as an axon guidance cue, is instead required for presynaptic assembly in AIY (Colon-Ramos, 2009; Colon-Ramos et al., 2007).

In addition, to determine cell autonomy and to clarify the function of UNC-40, a genetic fragment containing wild type *unc-40* was injected into *unc-40* loss-of-function mutants. The genetic fragment rescued the presynaptic vesicle phenotype in AIY only when expressed in AIY; presence of wild type UNC-40 in RIA did not rescue the presynaptic vesicle phenotype in AIY. Similarly, RIA exhibited normal axon guidance only when wild type UNC-40 was expressed in RIA. Thus, UNC-40 cell-

autonomously interprets the extracellular Netrin cue in AIY and RIA. Therefore, in one neural ciruit, Netrin and UNC-40/DCC are cell-autonomously affecting guidance and presynaptic assembly in RIA and AIY, respectively (Colon-Ramos, 2009; Colon-Ramos et al., 2007).

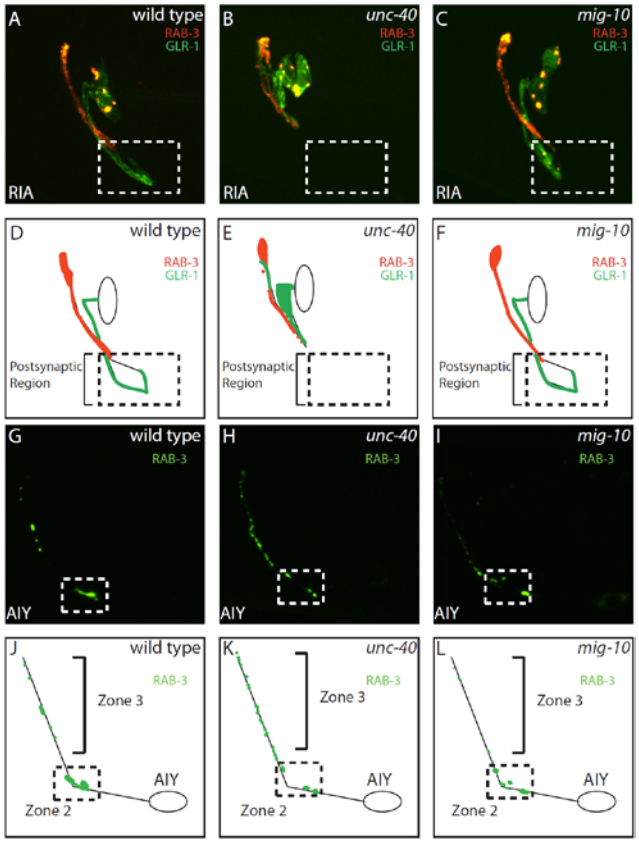

Figure 3. Genetic separation of UNC-40 and MIG-10 in AIY and RIA. (A-F) The guidance of postsynaptic RIA in wild type (A, D), *unc-40* mutants (B, E) and *mig-10* mutants (C, F). Compare A and C to note that RIA projects normally in *mig-10* mutants. Dashed boxes enclose the region that synapses with AIY. (G-L) Presynaptic assembly in AIY in wild type (G, J), *unc-40* mutants (H, K) and *mig-10* mutants (I, L). Compare H and I to note that *mig-10* is required for presynaptic assembly in AIY. Dashed boxes enclose Zone 2 region of AIY.

Recently, we determined that MIG-10 participates in the presynaptic assembly pathway in AIY. Loss-of-function *mig-10* mutants exhibited a synaptic vesicle phenotype in AIY similar to that of *unc-40* loss-of-function mutants (Fig. 3, compare I, L to H, K). However, RIA axon guidance was not disrupted in *mig-10* loss-of-function mutants, demonstrating a genetic separation of the pathways (Fig. 3C, F). Thus, Netrin instructs axon guidance in RIA through an uncharacterized mechanism. In constrast, Netrin is instructing presynaptic assembly in AIY through MIG-10.

Explaining how synaptic specificity is regulated at the molecular level during innervation of two *C. elegans* interneurons will provide a conceptual framework for understanding how more complex neural circuits are organized with incredible precision. The components of the axon guidance and synaptogenesis machinery are highly conserved across metazoans; thus, my findings could be applicable to vertebrate and human neurodevelopment. Our research indicates that while Netrin classically functions as an axon guidance cue in the postsynaptic interneuron RIA, Netrin acts in a novel role in AIY by influencing presynaptic assembly (Colon-Ramos et al., 2007). We determined that Netrin is affecting AIY:RIA innervation through MIG-10 in presynaptic AIY, but MIG-10 does not affect RIA guidance (Fig. 3). Study of the signaling pathway between Netrin and presynaptic assembly can be organized into three general questions. First, which MIG-10 isoform is involved in presynaptic assembly downstream of UNC-40 in AIY? Second, how is the Netrin signal transduced from UNC-40 to MIG-10? Identifying which molecules genetically interact between UNC-40 and MIG-10 in AIY will be important in explaining how Netrin instructs presynaptic assembly in AIY. Third, how does MIG-10 instruct presynaptic assembly? I will elucidate the mechanisms downstream of MIG-10 that connect this signaling module to synapse formation.

Research Design and Methods

I. Which MIG-10 isoform is involved in presynaptic assembly downstream of UNC-40 in AIY?

Due to the *mig-10* loss-of-function mutant phenotype, we determined that MIG-10 acts in presynaptic assembly in AIY (Fig. 3). To determine where MIG-10 is acting to instruct presynaptic assembly, we examined MIG-10 localization in AIY. As MIG-10 has three isoforms in *C. elegans*, we examined the localization of all three isoforms in AIY.

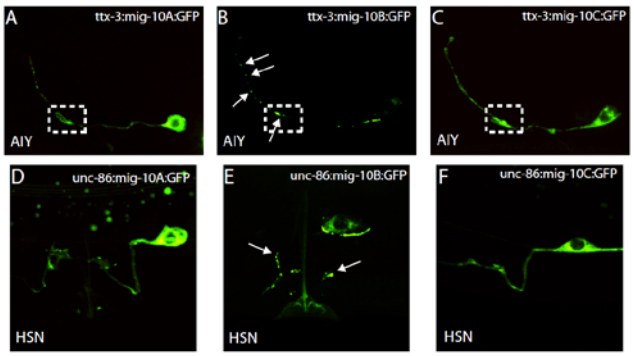

Figure 4. MIG-10 isoform localization. In AIY (A-C), MIG-10A::GFP (A) and MIG-10C::GFP (C) are cytoplasmic while MIG-10B::GFP localizes to synapses (B). Dashed boxes enclose Zone 2 of AIY (A-C). In HSN (D-F), MIG-10A::GFP (D) and MIG-10C::GFP (F) exhibit cytoplasmic signal while MIG-10B::GFP localizes to synapses (E). Arrows point to synaptic localization (B, E).

Previous studies indicate that the MIG-10 A isoform (MIG-10A) localizes to the HSN neuron growth cone early in development to mediate axon guidance (Adler et al., 2006; Chang et al., 2006; Quinn et al., 2008). To determine where MIG-10 is acting in AIY, GFP was fused in-frame to each *mig-10* isoform cDNA driven by the AIY-specific promoter *ttx-3*. We observed that MIG-10B::GFP localizes to presynaptic sites in AIY while MIG-10A::GFP and MIG-10C::GFP are cytoplasmic throughout AIY with no obvious subcellular localization (Fig. 4A-C). Localization of MIG-10B was disrupted in *unc-40* mutants, which suggests that MIG-10 acts downstream of UNC-40. Similar results were observed in HSN (Fig. 4D-F).

Since only MIG-10B localizes to synapses, we decided to determine which MIG-10B domains are required for MIG-10B localization. The three MIG-10 isoforms are nearly identical; MIG-10B has only one small unique exon that is not found in MIG-10 isoforms A or C. I tested whether the MIG-10B unique exon instructs synaptic localization. I determined that a MIG-10B unique exon:GFP fusion construct did not localize to synapses in AIY (data not shown). This result indicates that the unique exon is not sufficient for synaptic localization of MIG-10B; it may be required in combination with other MIG-10 domains, such as the RA domain that interacts with Rac GTPases (Fig. 5A).

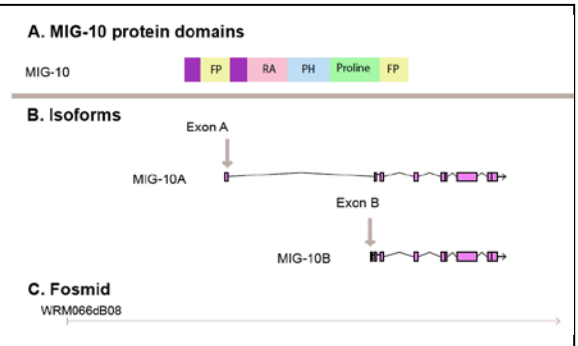

Figure 5. Schematic of MIG-10 structure. MIG-10 has 5 protein domains shared by all isoforms (A). MIG-10 A and B isoforms each have unique exons at the N-terminus, labeled with arrows (B). *C. elegans* fosmid WRM066dB08 contains MIG-10 isoforms A and B but not all of isoform C (C).

Based on MIG-10 localization, I hypothesize that MIG-10B is required for correct presynaptic assembly in AIY. To establish MIG-10 isoform function in AIY, I will use recombinogenic engineering (recombineering) of a genetic fragment (a fosmid) containing both *mig-10a* and *mig-10b*. First, I will replace the *mig-10b* unique exon with the mCherry fluorophore to disrupt only MIG-10B expression while MIG-10A expression and function are maintained (Fig. 5B). This construct will be microinjected into *mig-10* loss-of-function mutants and

scored for rescue of the Zone 2 *mig-10* synaptic vesicle phenotype. I will independently disrupt MIG-10A expression by replacing the MIG-10A specific exon with the mCherry fluorophore while maintaining MIG-10B expression and function. The construct will be microinjected into *mig-10* mutant animals and scored for rescue as described above. Analysis of the constructs' ability to rescue in HSN will serve as a control; the HSN *mig-10* mutant phenotype can be rescued by cell-specific expression of MIG-10A (Adler et al., 2006; Chang et al., 2006). As the Colón-Ramos lab has previously used recombineering successfully, I do not foresee any problems with this experiment.

The experiments proposed in Specific Aim 1 are designed to determine MIG-10 isoform localization and function in AIY. Understanding which MIG-10 isoform acts in presynaptic assembly will be important in explaining how MIG-10 instructs presynaptic assembly in AIY.

II. Which molecules mediate the genetic interaction between UNC-40/DCC and MIG-10/Lpd to transduce the Netrin signal in AIY?

Understanding how UNC-40 genetically interacts with MIG-10 will reveal how Netrin instructs presynaptic assembly in AIY. We demonstrated that both UNC-40 and MIG-10 participate in the presynaptic assembly pathway in AIY (Fig. 3G-L) (Colon-Ramos et al., 2007). Previous research has established that UNC-40 activates CED-10/Rac to determine localization of MIG-10 during axon guidance and outgrowth (Chang et al., 2006; Lundquist et al., 2001; Quinn and Wadsworth, 2008; Round and Stein, 2007). The function of CED-10/Rac1 during presynaptic assembly is unknown. I hypothesize that UNC-40 genetically interacts with MIG-10 through the Rac pathway to instruct presynaptic assembly in AIY.

To determine whether Rac pathway participants function in presynaptic assembly in AIY, I analyzed loss-of-function mutants for the presynaptic vesicle phenotype in AIY. CED-10/Rac1 has previously been implicated in regulating the localization of MIG-10 in axon guidance (Quinn et al., 2008). However, the *ced-10/Rac1* loss-of-function mutant did not exhibit disruption of axon guidance in AIY. Instead, *ced-10/Rac1* mutants exhibit a mutant phenotype in AIY Zone 2 similar to *unc-40* mutants (Fig. 6A-B, D-E). Therefore, CED-10/Rac is required for presynaptic assembly in Zone 2. Next, I tested whether CED-10 instructs MIG-10B synaptic localization in AIY. The AIY-specific MIG-10B::GFP fusion construct in *ced-10* loss-of-function mutants exhibited GFP signal in the cell body and all zones of AIY, in contrast to the synaptic localization of the fusion construct in wild type animals (data not shown). Thus, CED-10 instructs MIG-10B localization and directs presynaptic assembly in AIY. I observe a similar loss of synaptic localization of MIG-10B::GFP signal in *unc-40* loss-of-function mutants (data not shown).

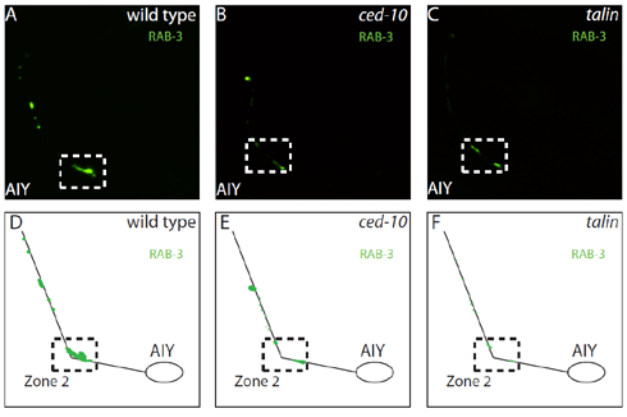

Figure 6. Putative participants in the AIY innervation pathway exhibit the AIY presynaptic phenotype. Presynaptic vesicles in wild type (A, D), *ced-10* (B, E) mutants and *talin* mutants (C, F). Compare (B, C, E, F) to Fig. 3 H, I, K, L to note phenocopy of *unc-40* mutants.

CED-10 GTPase activity is activated by two guanine nucleotide exchange factors (GEFs), UNC-73/Trio and CED-5/DOCK180, which genetically interact with UNC-40 (Fig. 1) (Lundquist et al., 2001; Quinn and Wadsworth, 2008). I hypothesize that the Rac GEFs instruct presynaptic assembly in AIY by affecting MIG-10B localization. To examine if Rac GEFs are required in presynaptic assembly in AIY, I will analyze *unc-73/Trio* and *ced-5/dock180* loss-of-function mutants for abnormal Zone 2 synaptic vesicle phenotype. Observation of an abnormal Zone 2 synaptic vesicle phenotype in GEF loss-of-function mutants would suggest that that they are required for presynaptic assembly in AIY. I will then analyze localization of the MIG-10B::GFP construct in GEF mutants to establish Rac GEF requirement for MIG-10B synaptic localization.

CED-10 is one of three Rac proteins in *C. elegans*. MIG-2 and RAC-2 are redundant with CED-10 in the axon guidance pathway (Lundquist et al., 2001). To understand the UNC-40:MIG-10 genetic interaction in AIY, I will determine whether the three Racs are redundant for presynaptic assembly in AIY or whether they act in separate pathways. I will first analyze the AIY synaptic vesicle phenotype of *rac-2* and *mig-2* loss-of-function mutants. If these Rac proteins are redundant in presynaptic assembly in AIY, I would expect single Rac mutants to exhibit less drastic synaptic vesicle phenotypes than *unc-40* mutants. If the single mutants have a synaptic vesicle phenotype in AIY, they are at least partially required for presynaptic assembly in AIY. However, if *mig-2* and *rac-2* mutants do not exhibit a synaptic vesicle phenotype, they may act redundantly or they may not function in presynaptic assembly. To determine whether they are redundant in the AIY presynaptic assembly pathway, I will also generate double and triple Rac mutants. If they are redundant, the double and triple mutants will exhibit more drastic phenotypes than any of the single mutants, including the *ced-10* mutant.

The experiments proposed in Specific Aim 2 are designed to inform our understanding of the UNC-40:MIG-10 genetic interaction in during presynaptic assembly.

III. Which molecular pathways are activated by MIG-10 to direct presynaptic assembly in AIY?

Because we have identified a novel function for MIG-10, the pathway downstream of MIG-10 in presynaptic assembly is uncharacterized. I propose to identify the mechanisms downstream of MIG-10 that instruct presynaptic assembly in AIY.

There are two known effectors downstream of MIG-10: UNC-34/Enabled and Talin (Chang et al., 2006; Krause et al., 2004; Lee et al., 2009). We have analyzed UNC-34 function with several assays, and we have no evidence that UNC-34 is involved in presynaptic assembly in AIY. When we examined a *talin* mutant in AIY, we observed no axon guidance defects. However, the *talin* mutant is required for presynaptic assembly in Zone 2 (Fig. 6 C&F). A previous study demonstrated that Talin binds to the N-terminus of RIAM, a MIG-10 homolog (Lee et al., 2009). A separate study indicates that Talin is enriched at presynaptic sites (Morgan et al., 2004). The MIG-10B unique exon contains an N-terminal region similar to that of RIAM. Thus, I hypothesize that MIG-10B binds Talin to regulate synaptic assembly in AIY. I will perform genetic analysis to establish Talin's involvement downstream of MIG-10. Epistasis experiments require **two null mutants** to analyze the interaction between two genes. However, there is no null *talin* mutant. Thus, I will generate a null *talin* allele in a screen of a deletion mutant library from Dr. Michael Koelle's lab (Ahringer, April 6, 2006). Double mutant studies with *mig-10;talin* will determine whether Talin acts downstream of or parallel to MIG-10. If the double mutant exhibits a stronger synaptic vesicle phenotype than either of the single mutants, Talin and MIG-10 participate in parallel

pathways. I would then generate double mutants with Talin, UNC-6/Netrin and UNC-40/DCC. These subsequent epistasis experiments would reveal whether Talin participates downstream of UNC-40/DCC.

Previous studies implicated Talin localization at presynaptic sites (Morgan et al., 2004). I hypothesize that Talin acts downstream of MIG-10 in presynaptic assembly , resulting in Talin localization to presynaptic sites in AIY. Talin::GFP fusion constructs in colocalization studies with known synaptic proteins will reveal the subcellular localization of Talin in AIY. Observation of these fusion constructs in *mig-10*, *unc-40*, *unc-6*, and other null mutants will elucidate whether Talin subcellular localization is dependent on, and downstream of, those proteins. My results suggest that Talin functions in presynaptic assembly (Fig. 6C&F). Synaptic localization studies will inform our understanding of Talin in AIY. Synaptic localization of Talin would suggest that Talin may be downstream of MIG-10 in presynaptic assembly in AIY. I must note that a lack of synaptic localization of Talin would not undermine my hypothesis that Talin acts downstream of MIG-10; cytoplasmic localization does not rule out localized protein function.

It is possible that Talin is not downstream of MIG-10 and that other molecules act downstream of MIG-10 in presynaptic assembly in AIY. To address this possibility and to discover any novel participants in the presynaptic assembly pathway, I will perform a forward genetic screen. A forward genetic modifier screen by EMS mutagenesis with the *talin* mutant will likely identify molecules that affect presynaptic assembly in AIY and are acting in the same pathway as or in parallel to Talin.

The experiments proposed in Specific Aim 3 are designed to determine the molecular pathway downstream of MIG-10 in the presynaptic assembly module in AIY.

The completion of these experiments will inform out understanding of how Netrin instructs presynaptic assembly in AIY. Revealing this mechanism will provide a conceptual framework for Netrin organization of AIY:RIA innervation and, more generally, understanding synaptic specificity and proper formation of neural circuits.

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