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The Effects of Deletion of the Cytoplasmic Domain of Robo3 on Drosophila

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The Effects of Deletion of the Cytoplasmic Domain of Robo3 on Drosophila

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Introduction

As organisms with nervous systems form and develop, their neurons lengthen and grow. Neurons connect with each other by sending out axons during development. An axon is an extension of the neuron's membrane. Axons convey information from the nerve cell body to the nerve terminal (Debanne et al., 2011).

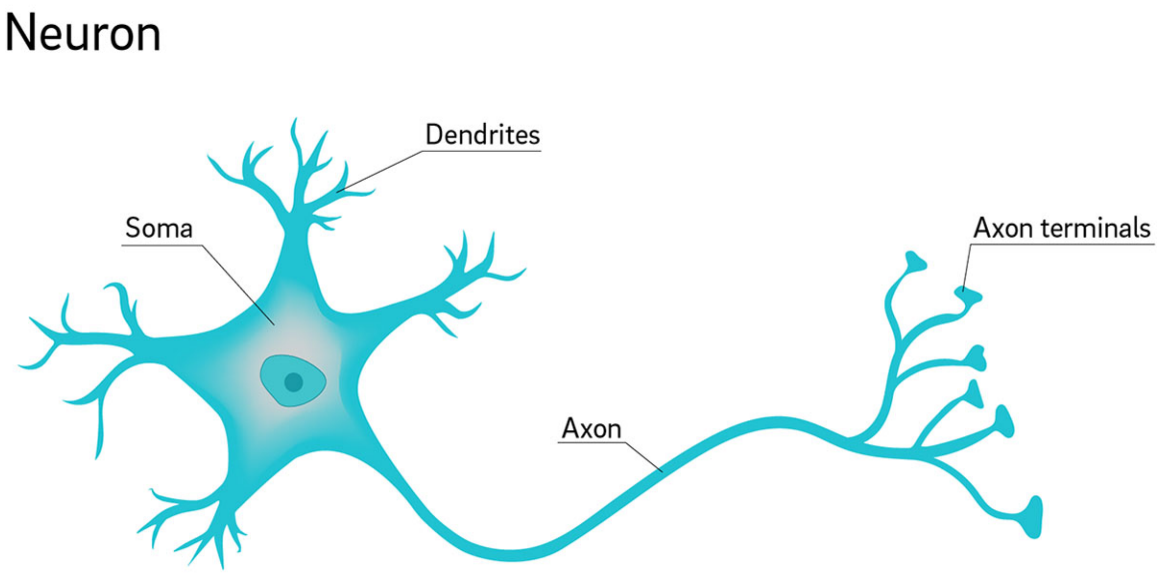

Figure 1.

A model of a neuron (Connor 2018).

The formation of axons is essential in organisms with a nervous system. Axon guidance helps during the development of the nervous system. Axons can reach many parts of the body. To reach their target, axons are guided during the development of the embryo by extracellular signals. These signals are sensed by axon guidance receptors. Axon

guidance directs axons either across the midline of the nerve cord, away from the midline, or along the edge of the nerve cord (Evans and Bashaw, 2010). After axons are guided to their target, axon guidance also keeps axons from deviating from their targeted pathway. An important group of axon guidance receptors is the Roundabout (Robo) family. The role of the Robo receptors is to regulate midline crossing of axons in a wide range of animals (Parsons et al., 2003). It does this by signaling midline repulsion in response to the binding protein, Slit. The Slit protein is currently the only known ligand for the Robo family receptors (Kidd et al., 1999)

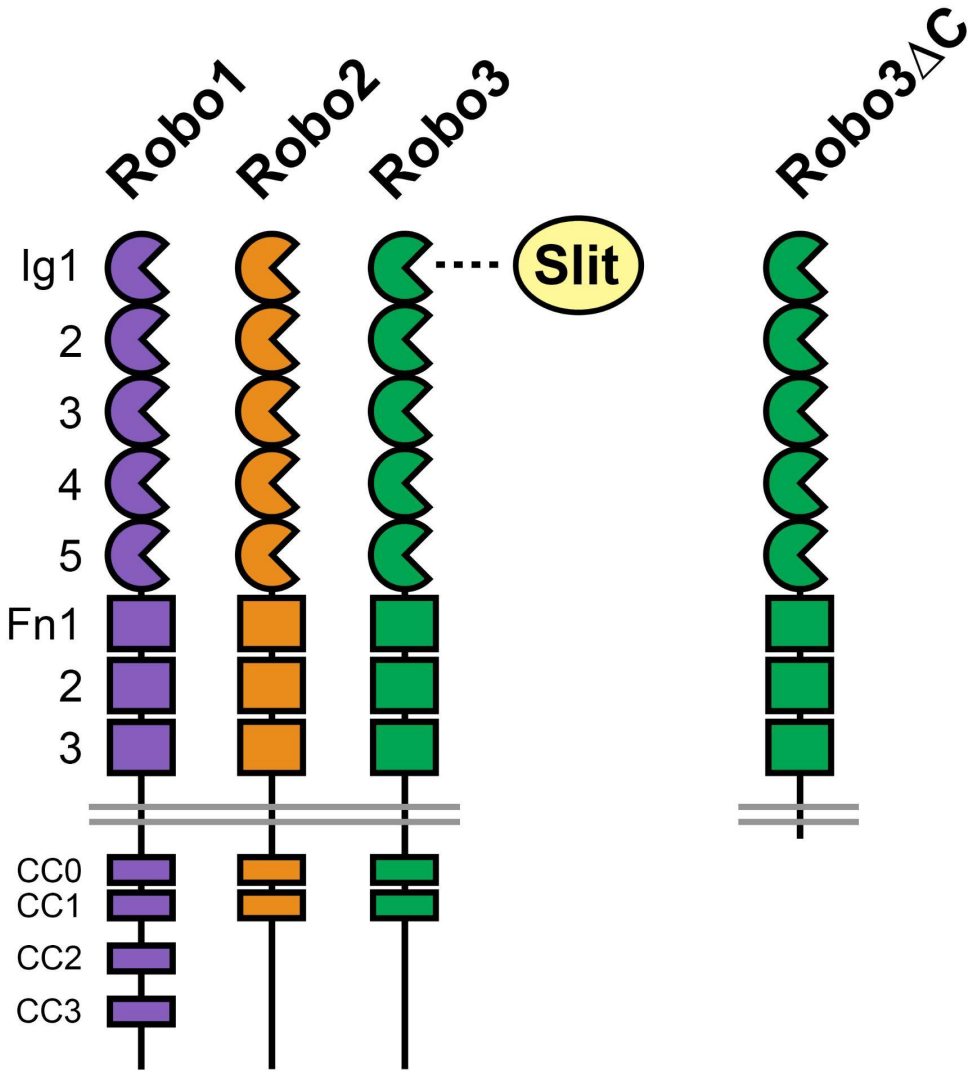

Figure 2.

A model of the Roundabout family receptors. Each Robo receptor has five immunoglobulin-like (Ig) domains and three fibronectin (Fn) domains. Robo1 has four different cytoplasmic (CC) motifs, with Robo2 and Robo3 having two CC motifs. The robo3ΔC variant is also shown. The full-length Robo3 protein is 1,342 amino acids long. We deleted 443 amino acids from the cytoplasmic domain of Robo3.

Previous research focuses on the importance of the Robo family in *Drosophila*. The Robo family in *Drosophila* consists of three different family members, Robo1, Robo2, and Robo3. It has been found that the Slit ligand binds to the Immunoglobulin1 (Ig1) domains of all Robo paralogs. The removal of the Ig1 domain in Robo1 prevents Slit from binding to Robo1 (Brown et al., 2015). It has also been recently found that the removal of the Ig1 domain in Robo2 stops midline repulsion due to Slit being unable to bind (Howard et al., 2021).

Robo3 in *Drosophila* plays an important role in the formation of intermediate axon pathways. It is expressed along the embryonic ventral nerve cord and is thought to regulate axons in the intermediate region away from the midline of the neuropile (Rajagopalan et al., 2000). The function of Robo3 in guiding axons is evolutionarily conserved in insects. Robo3 in *Drosophila* has been replaced using the Robo2/3 receptor from *Tribolium castaneum*, the flour beetle. Normal expression was found in the replaced receptors showing that Robo2/3 in *Tribolium* performs the same function as Robo3 in *Drosophila* (Evans 2017). In this role, Robo3 is hypothesized to act as a SLIT receptor directing axons further from the midline by signaling in response to SLIT. Robo3 is made up of five different Ig domains, three fibronectin (Fn) domains, and two cytoplasmic

motifs (CC). It is known that the Ig1 domain of Robo3 is needed for Slit binding. Research is currently being conducted in Dr. Evans's lab to examine the function of the other domains.

My research project examines how the deletion of the cytoplasmic domain affects the function of Robo3. If Robo3 is signaling repulsion in response to SLIT this activity should require the cytoplasmic domain. I investigated the functional importance of Robo3 by deleting the cytoplasmic domain of Robo3 using a CRISPR-based technique. This modified gene was then injected into *Drosophila* embryos where it replaced the normal copy of the gene. Embryos expressing the modified version of *robo3* in place of normal *robo3* were dissected and examined.

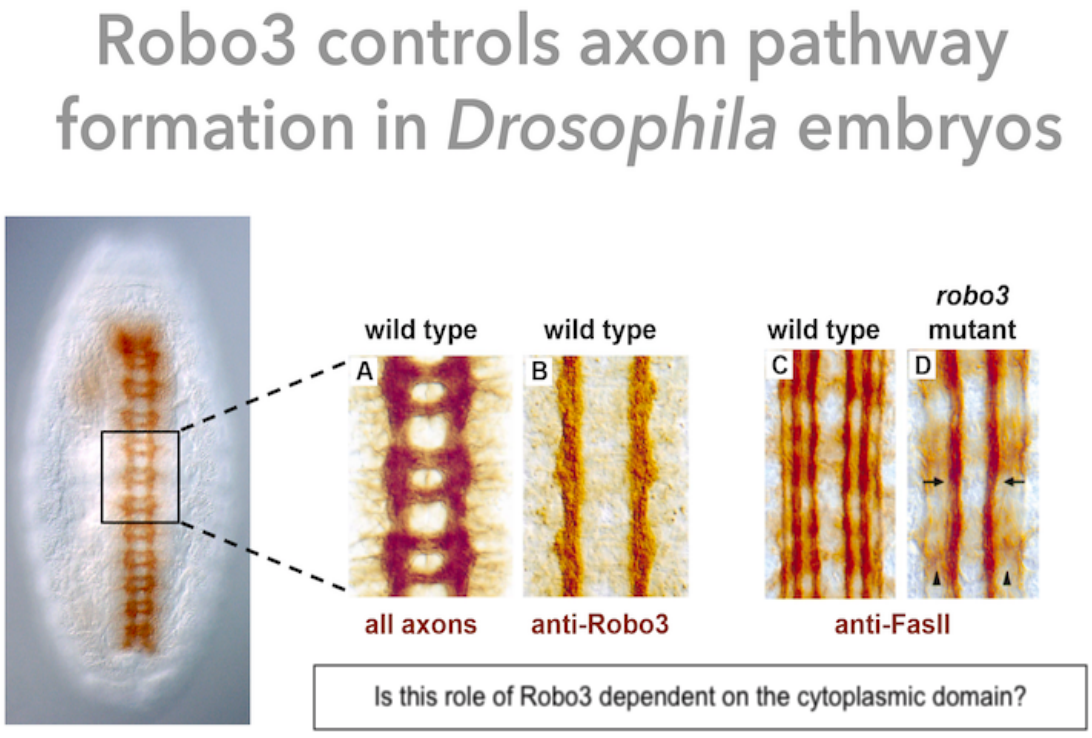

Figure 3.

Left, Drosophila embryo stained with antibody to label all of the axons in the central nervous system (brown stain). ***A)*** *shows a magnified view of three full segments of the nerve cord in a wild-type embryo.* ***B)*** *Robo3 protein is localized to longitudinal axon pathways in wild-type embryos.* ***C)*** *An antibody against the Fasciclin II protein (anti-FasII) labels three distinct longitudinal pathways on each side of the midline in wild-type embryos.* ***D)*** *In robo3 mutant embryos, the intermediate FasII pathway fails to form in most hemisegments (arrows). Therefore, Robo3 is required for the formation of intermediate axon pathways. We do not yet know if this function of Robo3 depends on its cytoplasmic domain. (Simpson et al., 2000) (Rajagopalan et al., 2000)*

Methods

Construction of *robo3* donor plasmid

The construction of the *robo3*$^{robo3\Delta C}$ donor was done using a quick change reaction based on an already made template donor. The previously made donor had targeted guide RNAs (gRNA) that created a double-strand break at the 5' and 3' ends of *robo3*. The double-strand breaks were repaired using the homologous *robo3*$^{robo3\Delta C}$ donor plasmid. The resultant coding sequence replaced the original *robo3* sequence with the robo3ΔC complementary DNA (cDNA).

Fly Crosses

The following *Drosophila* strains were used: yellow, white (yw), *Sco/CyOwg*, *robo3*^{robo3}, and *robo3*$^{robo3\Delta C}$. Over 300 embryos were injected with the donor plasmid and

robo3 gRNAs, and 110 survived. Injected adults (G0) were crossed with *Sco/CyOwg* flies. All crosses were carried out at 25 °C. Founder adults (G0 flies whose F1 progeny carried the modified robo3 alleles) were identified by collecting two pools of three F1 females from the G0 crosses and tested with PCR primers oligo 589 and oligo 591. The expected result of those with the robo3ΔC allele was about 1.6-kbp. Ten F1 males were taken from each of the three positive G0 lines and crossed individually with *Sco/CyOwg* virgin females. The F1 males were then removed and tested for the modified allele through PCR using the same oligo primers. F2 flies from positive F1 crosses were used to create balanced stocks. The modified alleles were then fully sequenced by amplifying the entire modified locus (approx. 6 kb) from genomic DNA using oligo primers 589 and 590. The PCR product was then sequenced after cloning via CloneJET PCR cloning kit (Thermo Scientific) (Evans 2017).

Imaging

Drosophila embryo collection, fixation, and antibody staining were carried out as previously described (Patel, 1994). The following antibodies were used: mouse anti-HA(BioLegend #901503, 1:1000), mouse anti-βgal (Developmental Studies Hybridoma Bank [DSHB] #40-1a, 1:150), goat anti-HRP 488 (Jackson Immunoresearch #123-545-021, 1:500), goat anti-mouse Cy3 (Jackson #115-165-003, 1:1000), and mouse anti-FasII (DSHB #1D4, 1:100). Embryos were genotyped through balancer chromosomes that carried lacZ markers. The ventral nerve cords of embryos of the desired developmental stage and genotype were dissected and mounted on a slide with 70% glycerol/PBS. Fluorescent confocal stacks were collected using a Leica SP5

confocal microscope and processed by Fiji/ImageJ and Adobe Photoshop software (Evans 2017).

Results

CRISPR/Cas9-based gene replacement of *Drosophila robo3*

The deletion of the cytoplasmic domain of Robo3 was carried out through gene modification using CRISPR/Cas-9 (Gratz et. al, 2014). A previously made *robo3* coding sequence ($robo3^{robo3}$) was used to create the donor plasmid ($robo3^{robo3\Delta C}$). The $robo3^{robo3}$ template was made in 2016 and can be used to create alternative coding sequences for Robo3 using homology-directed repair. The $robo3^{robo3\Delta C}$ donor was tested through PCR testing with an expected size of about 7.7-kbp. It was also tested using DNA sequencing. The $robo3^{robo3\Delta C}$ donor plasmid and two *robo3* gRNAs were injected into *Drosophila* embryos.

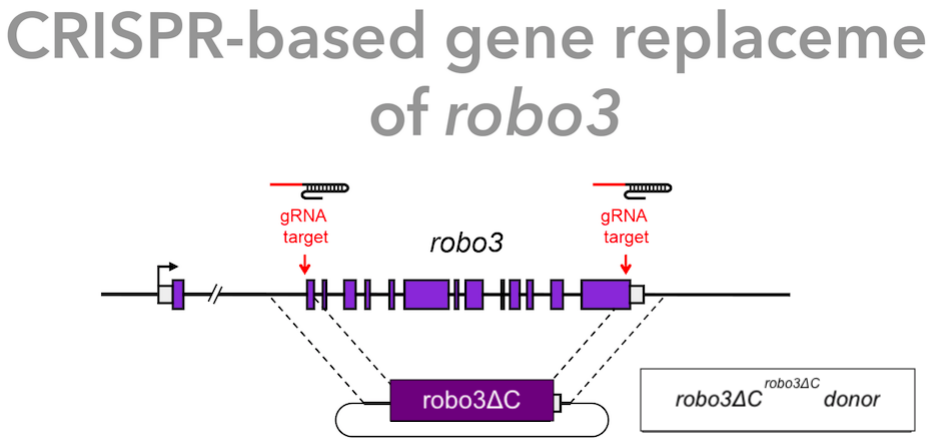

Figure 4.

CRISPR/Cas-9 mediated replacement of the robo3 gene. Two guide RNAs localize Cas9-induced double-strand breaks to targets flanking the robo3 coding region, and homology-directed repair replaced this region with an HA-tagged robo3ΔC complementary DNA.

The *robo3ΔC* allele alters the protein localization of Robo3

In order to examine the $robo3^{robo3\Delta C}$ allele, I first compared its expression to normal Robo3 protein expression in wild-type and modified genetic strains. Axons that express Robo3 usually form or join longitudinally in the lateral two-thirds of the neuropile. Wild-type Robo3 is undetectable in the CNS to hemagglutinin (HA) staining, so a modified $robo3^{robo3}$ allele was used where most of the Robo3 coding exons and intervening introns were replaced with an HA-tagged $robo3^{robo3}$ cDNA (Evans 2017). All embryos were also stained with an antibody against horseradish peroxidase (anti-HRP) which labels all of the axons in the ventral nerve cord.

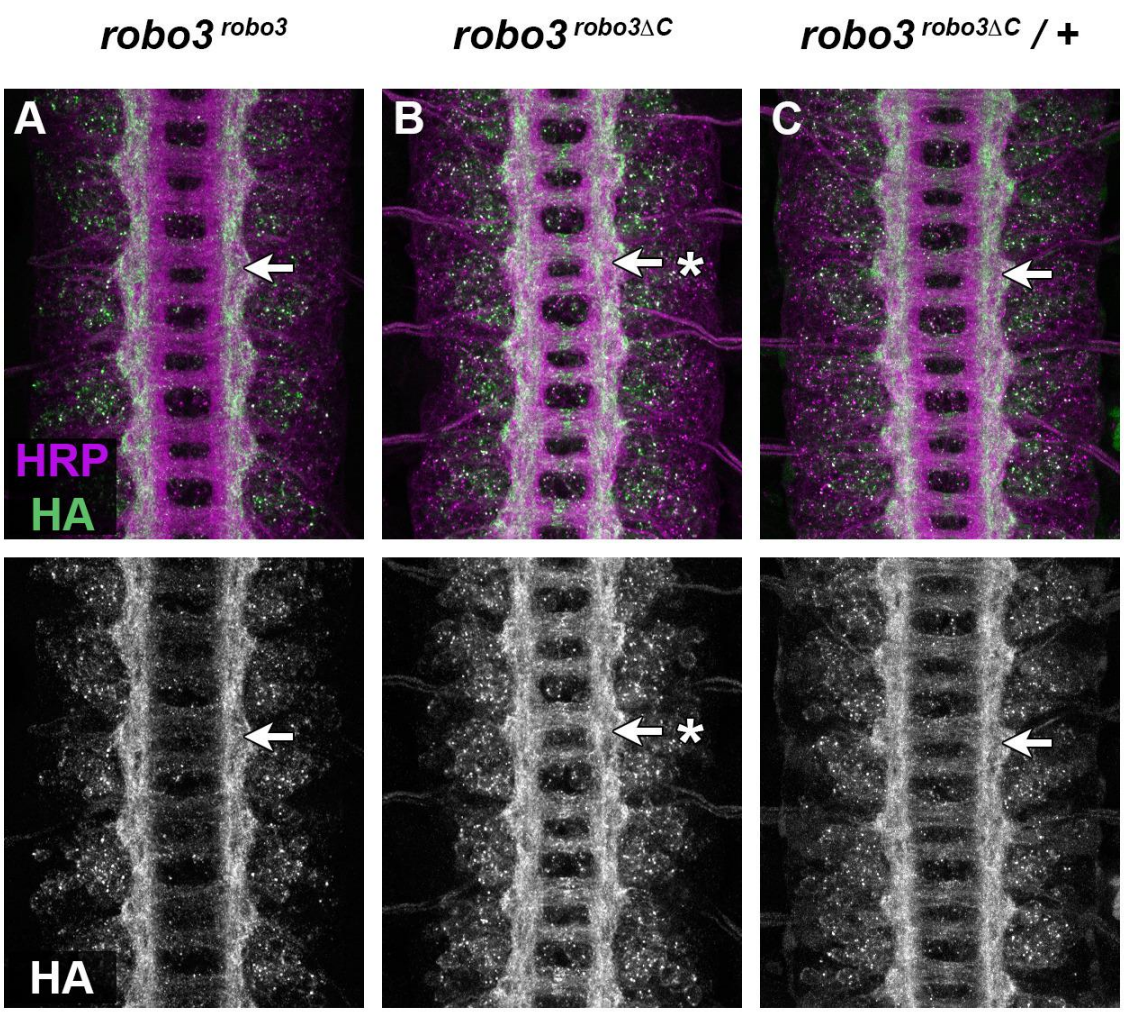

Figure 5.

HA expression in modified robo3 alleles. ***A)*** *A full-length robo3 replacement allele, HA expression reproduces the wild-type Robo3 protein expression (arrow).* ***B)*** *The HA-tagged robo3ΔC is detectable on both longitudinal and commissural (the midline sections) axons. The robo3ΔC protein doesn't appear to be restricted to the lateral two-thirds of the axon scaffold (arrow and asterisk).* ***C)*** *A modified heterozygous robo3 allele containing both the robo3ΔC and wild-type alleles. The HA-tagged protein shows more expression in the commissural axons than the control allele but not as much as the robo3ΔC protein.*

When I compared the HA staining of the control full-length replacement embryos to embryos of homozygous *robo3ΔC* I saw a number of differences. I saw increased cell body staining. Displayed as the increased levels of white around the outside of the embryo. There were also increased levels of HA staining on midline crossing commissural axons, seen as the brighter levels of white in the midline crossing sections compared to the control. Finally, the expansion of HA into the medial zone of the longitudinal axons for the *robo3ΔC* allele compared to the control. The thicker white axon scaffolds formed closer to the middle of the embryo.

The *robo3ΔC* modified allele displays partial protein function

To further examine the function of *robo3ΔC*, I used an antibody against Fascilin II (FasII) to look at the formation of longitudinal axon pathways in wild-type and mutant *robo3ΔC* gene replaced embryos. After staining the embryos, I then quantified the different phenotypes. I examined the intermediate FasII pathways of embryos with different genotypes and scored whether they formed correctly or not. The scored embryos were then quantified and the percentage of defects in embryos with different genotypes was calculated.

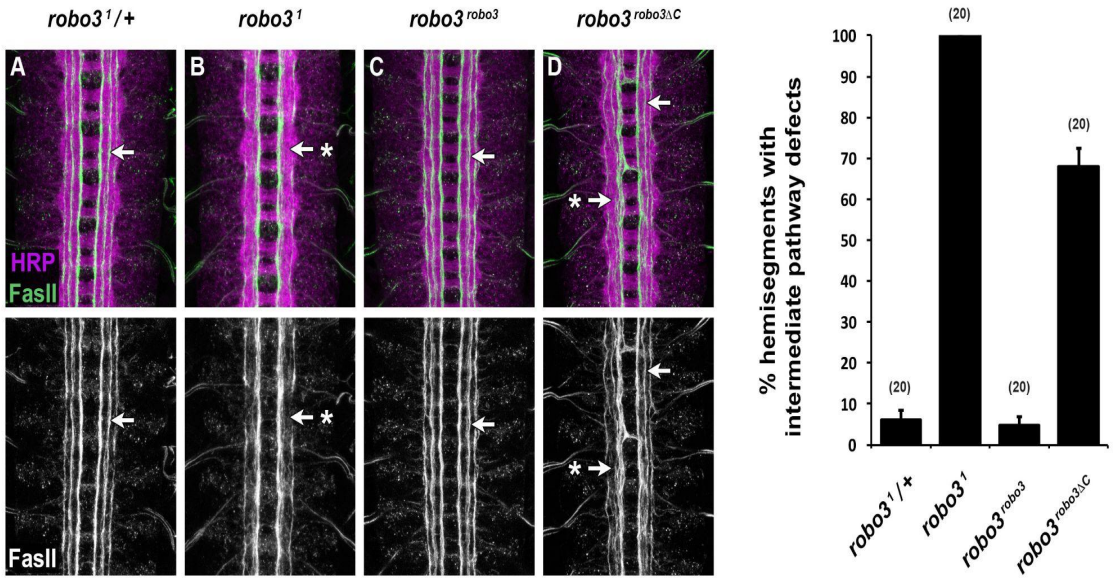

Figure 6.

In wild-type Drosophila embryos, FasII-positive axons form three distinctive longitudinal pathways on either side of the midline and a distinct intermediate pathway is visible in every hemisegment ***(A, arrow)****. In robo3 loss-of-function mutants, the intermediate pathway fails to form* ***(B, arrow with asterisk)****. In embryos with full-length replacement, the pathways formed are the same as in wild-type embryos* ***(C, arrow)****. In embryos where robo3 has been replaced with robo3ΔC, the intermediate pathway forms in around 30% of hemisegments* ***(D, arrow)****. However, it is defective in the other 70% of hemisegments* ***(D, arrow with asterisk)****. The bar graph quantifies intermediate pathway defects in the genotypes shown* ***(A-D)****. Error bars represent the standard error of the mean.*

I found that *robo3ΔC* embryos exhibited axon guidance defects that were worse than the control embryos, but not as bad as the loss-of-function *robo3* mutants. I concluded from this that *robo3ΔC* had retained some of its function but it doesn't work as well as the full-length protein.

Discussion

In this project, I examined the functional importance of the cytoplasmic domain of Robo3. I did this by using a modified allele where the cytoplasmic domain was deleted. I found that this deletion altered the localization of the protein, the protein also had partial rescue in axon guidance function.

I think that the change in protein localization in the *robo3ΔC* allele is due to the fact that the Robo3 protein-expressing axons are misguided into the medial zone. This can be seen in the partial rescue of the axon guidance function as well. Where some, but not all of the intermediate axons are misguided into the medial zone. The protein moving closer to the midline is not due to the fact that the protein itself is localized to the midline. The domain of Robo3 expression expands, no longer to just the later two-thirds of the axon scaffold, but into the medial zone. That means that some of the axons that are normally excluded from that zone due to the function of Robo3 can move in. As those axons move closer to the midline, it carries the protein with them, and we see HA in the zone where it is not typically found.

A previous experiment in Dr. Evans's research lab looked at the effects of the deletion of the Ig1 domain in Robo3. It was found that the protein was also mislocalized in vivo however the mislocalization of those embryos was worse than the protein mislocalization of this project. The embryos, similar to this project, also still had partial function of Robo3. That project and this one are currently the only two known deletions made to Robo3 domains. Both projects have found that Robo3 still has some activity

after deletion but not full function. In comparing the deletions of Robo3 domains to both deletions of Robo1 and Robo2 Ig1 domains, only Robo3 has some partial function. The deletion of the Ig1 domain in both Robo1 and Robo2 resulted in a complete loss of function (Brown et al., 2015; Howard et al., 2021). This indicated that the Roundabout proteins are not acting equivalent to one another.

The partial rescue of the Robo3 function indicated that no matter what happens with the protein being mislocalized, there is still some function. When the Ig1 domain of Robo2 is deleted, all function of the protein was completely lost and the protein was mislocalized (Howard et al., 2021). A conclusion couldn't be drawn as to which caused which. There could potentially be a complete loss of function due to the mislocalization of the protein or the protein could have been misguided due to the loss of function. In this project, while the protein is not completely localized properly, some function still remains. That could allow us to conclude that there's something that Robo3 executes that doesn't require the cytoplasmic domain and suggests that Robo3 may regulate the lateral position of axons through a different mechanism than SLIT-dependent repulsion.

We can conclude that the cytoplasmic domain is partially required for the function of Robo3 from this project. In future investigations, I think the next logical step would be to examine the cytoplasmic domain closer. The cytoplasmic domain of Robo3 is made up of two motifs, CC0 and CC1. The same gene replacement used in this project can be used to target the two motifs separately. One could delete only the CC0 motif, then delete only the CC1 motif, and examine the results of those deletions. If one particular motif has a stronger effect on function than the other.

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