The dual role of the ligand UNC-6/Netrin in both axon guidance and synaptogenesis in C. elegans

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Abstract: The extracellular cue UNC-6/Netrin is a well-known axon guidance molecule and recently it has also been shown to be involved with localization of pre-synaptic complexes. Working through the UNC-40/DCC/Fra receptor, UNC-6/Netrin promotes the formation of pre-synaptic terminals between the pre-synaptic AIY interneuron and its post-synaptic partner, the RIA interneuron. In the DA9 motor neuron, UNC-6/Netrin has an alternate role promoting the exclusion of pre-synaptic components from the dendrite via its UNC-5-receptor. Surprisingly, the requirement for UNC-5 persists even after DA9 axon migration is complete, because synapses become mis-localized after it is depleted. This observation provides at least a partial explanation for the persistence of UNC-6/Netrin and UNC-5 in the adult nervous system. These activities parallel the previously known bi-functional axon guidance effects of UNC-6/Netrin, since it can attract cells and axons expressing UNC-40/DCC/Fra and repel those expressing UNC-5 alone or in combination with UNC-40. UNC-6/Netrin cooperates with the Wnt family members to exclude synapses from compartments within the DA9 axon, so that they only occur in regions free of the influence of both UNC-6/Netrin and the Wnts. Regulation of both axon guidance and synapse formation by axon guidance cues permits coordination in circuit assembly between pre- and post-synaptic cells.

During development of the nervous system, differentiated progenitor cells become polarized and send out processes from the cell body that later become dendrites and axons. The pro-neural cells themselves and later their axons, often migrate long distances to their eventual targets using guidance cues. Once the destination is reached, the axon usually selects among several available targets and establishes synapses with the correct post-synaptic partner. The synapse is the site of communication between the pre- and post-synaptic cell and many of the molecules involved in synapse formation are known. Development of both the pre- and post-synaptic cells needs to be orchestrated to ensure that they are available to form synapses with each other and this process can be directed by guidepost cells. In organisms such as vertebrates, the guidepost cells are often glia, which guide two neurons to ensure that the correct synapse is formed. A case is presented here where glial cells secrete a cue to control the localization of pre-synaptic complexes in C. elegans. One notable aspect of this process is that the glial-secreted cue is a well-known axon guidance molecule, namely Netrin/UNC-6, but here it plays an additional and surprising role in selecting the site for the construction of a pre-synaptic complex.

UNC-6/Netrin, is a well-known bi-functional axon guidance cue that can attract some axons and repel others. It is a laminin-related molecule, originally isolated from C. elegans, with homologues in higher organisms. UNC-6 has two receptors in C. elegans: UNC-40 and UNC-5. Both have homologues in higher organisms: UNC-40/DCC/Fra (Deleted in Colorectal Cancer in vertebrates/Fra zzled in Drosophila) and UNC-5/Unc5. UNC-6/Netrin is expressed by cells mostly located in the ventral regions of C. elegans where it attracts many cells and axons expressing the receptor UNC-40. Conversely, UNC-6/Netrin repels axons and cells expressing UNC-5 alone, or in combination with UNC-40. One aspect of this developmental process, however, that has been somewhat puzzling has been the observation that expression of both UNC-6 and UNC-5 persist into adulthood. A partial explanation for the persistence of UNC-6 and UNC-5 is provided by Poon et al. who found that UNC-5 is required for both the initial polarized localization and maintenance of the pre-synaptic complexes in the DA9 motor neuron axon in C. elegans. The mechanisms used by the proteins in these new roles have not been established, but the localization of both UNC-5 and UNC-40 in the axons is controlled by their normal ligand, UNC-6/Netrin.
UNC-40/DCC/Fra plays two independent roles in establishing the connection between the pre-synaptic AIY amphid inter-neuron and its postsynaptic partner, the RIA inter-neuron in the nerve ring of C. elegans, since it is involved in both axon guidance of RIA and synapse localization in AIY.\(^{4}\) The two neurons are located in the head region, close to the nerve ring and can be visualized using cell-specific markers (Fig. 1). UNC-6/Netrin plays a conventional guidance role in directing migration of the post-synaptic inter-neuron RIA, since the ventral trajectory of its axon is altered in the absence of UNC-40. The axon of the AIY inter-neuron migrates anteriorly from its cell body, then dorsally and synapses onto three other interneurons: RIA, AIZ and RIB. The AIY axon usually migrates normally without the UNC-40 receptor, which is not surprising as it does not make a ventral migration.

Pre-synaptic complexes in AIY were detected by expression of fluorescently-tagged synaptic vesicle associated RAB-3.\(^ {4}\) They were found mainly in the "elbow" region (Fig. 1, zone 2) and about eight more complex-containing areas were found in the region most distant from the cell body within the nerve ring in wild-type animals (Fig. 1, zone 3): A hypomorphic allele of unc-40, wy81, was found in a genetic screen for mutants exhibiting altered localization of pre-synaptic complexes. In the absence of fully functional UNC-40, the pre-synaptic markers were not observed in zone 2, but were present in the more distal region, zone 3. In addition, the pre-synaptic region (zone 2) of AIY did not have an expanded diameter in the manner characteristic of this particular synapse, as detected by electron microscopy. The synapses between AIY and RIA in the absence of UNC-40 were abnormal in several other respects. There was a severe reduction in the active zone proteins ELKS-1/ERC/CAST and SYD-2/αLiprin, suggesting a defect in the pre-synaptic differentiation of AIY. Pre-synaptic defects in AIY caused by the absence of UNC-40 could only be rescued by cell-autonomous expression of the receptor.

Localization of UNC-40/DCC/Fra in the AIY interneuron is controlled by UNC-6/Netrin emanating from a pair of glial cells called the ventral cephalic sheath cells (VCSCs), which are similar to astrocytes.\(^ {4}\) Wadsworth et al.\(^ {11}\) have previously shown that the VCSCs at the nerve ring express UNC-6/Netrin during neurulation. Colon-Ramos et al.\(^ {4}\) found the VCSCs project deeply invaginated end-feet that form membranous lamellae, thereby ensheathing the region of AIY-RIA synapses. There is thus a very tight association between the glial cell and the two interneurons in the region of the synapses in zone 2. UNC-40 localizes to the pre-synaptic zones 2 and 3. In the absence of UNC-6, UNC-40 is more diffuse and is present along the entire neuron.

The anatomical relationship between the sheath cells and synapses is instructive in mediating AIY:RIA innervations. Sheath cell morphology was altered by the absence of UNC-34/Enabled such that the glial end-feet now migrated further posteriorly to include zone 1.\(^ {4}\) There was a concomitant appearance of both ectopic pre-synaptic complexes and UNC-40 localization in zone 1 due to an alteration in the source of UNC-6. In UNC-34/Enabled mutants, the trajectory of the RIA interneuron was also altered, such that it had migrated towards the new site of the synapses. Therefore, in this study UNC-40 is playing two independent roles, one in axon path-finding of the RIA axon and a second in positioning the synapses in the AIY pre-synaptic cell. Both of these activities are under the control of UNC-40’s normal ligand, UNC-6/Netrin, that is expressed by glial cells that ensheath the region of the synapses. Regulation of both processes by a single molecule allows co-ordination in circuit assembly.

In contrast to the work described above, UNC-6/Netrin and its receptor UNC-5 have recently been reported to exclude synaptic vesicle and active zone components from the dendrite of the DA9 motor neuron in C. elegans (Fig. 2).\(^ {14}\) The DA9 neuron can be divided into five zones (see insert in Fig. 2). It synapses en passant with the VD/DD motor neurons and the body wall muscles along the dorsal cord. In wild-type animals, the synapses of the DA9 neuron were detected using a fluorescently labelled RAB-3, a synaptic vesicle associated protein, and they were found mainly in the region most distant from the cell body (zone 5 of DA9 in Fig. 2). Synapses were entirely excluded from the dendrite (zone 1) and the remainder of the axon (zones 2, 3 and 4) in wild-type animals. Interestingly, UNC-5 had a somewhat complementary distribution to the pre-synaptic complexes since it was expressed mainly in the dendrite of DA9 and the ventral region of...
the axon (zones 1 and 2 in Fig. 2), suggesting that the presence of UNC-5 can exclude synapses.

The correct location of the pre-synaptic complexes in DA9 is dependent on both the ligand UNC-6/Netrin and the receptor UNC-5. RAB-3 was found ectopically in the dendrites of either unc-6(ev400), or unc-5(e53), both considered to be null mutants.5,9,15 Other pre-synaptic vesicle proteins tested, including SNB-1/Synaptobrevin and SNG-1/Synaptogyrin, as well as CCB-1, an L-type voltage-gated calcium channel β subunit and the active zone protein SYD-2/α-liprin, were also mis-localized in the dendrite in the absence of either UNC-6 or UNC-5.14 UNC-5 functions cell autonomously for the exclusion of pre-synaptic complexes. Interestingly, deletion of either one of the immunoglobulin domains or one of the thrombospondin domains from the extracellular regions of an UNC-5 protein was previously shown to alter the sub-cellular localization of the protein so that it was more localized to the cell body than wild-type UNC-5.13 This suggests that the extracellular region of UNC-5 is responsible for its localization in the neuron and it would be interesting to see if synapse localization is affected in the absence of the extracellular domains.

In addition to its roles in axon guidance and localizing pre-synaptic complexes, an ongoing supply of UNC-5 is required in DA9 to maintain the position of the synapses. This has been demonstrated by the use of a temperature-sensitive silencing intron construct that allowed UNC-5 expression at a permissive temperature of 25°C but not at the restrictive temperature of 16°C.14 Temperature shift experiments from the permissive temperature to the restrictive temperature at the L4 stage, after the axon was already fully developed, caused synapse mis-localization similar to that observed in the absence of UNC-5. Initial synapse mis-localization was irreversible as the reverse shift from the restrictive to the permissive temperature at L4 failed to rescue the defect. The exclusion of pre-synaptic complexes from all the compartments of DA9 except for the most distal regions (zones 4 and 5) was not simply a consequence of axon misguidance, since axons that were not misguided due to the absence of UNC-5, still exhibited altered RAB-3 localization. Additionally, animals lacking another axon guidance cue, UNC-129/TGFβ exhibited misguidance of DA9 but not mis-localization of pre-synaptic components. Dendritic localization of the pre-synaptic proteins was also not just a reversal of the axons and dendrites in DA9, since four different dendritic proteins were correctly localized in the absence of both UNC-6/Netrin and UNC-5. The need for an ongoing supply of UNC-5 accounts for the observation that both UNC-5 and UNC-6 persist into adulthood, long after axon guidance or synapse formation in worms.11,13 The finding that UNC-5 must be present on an ongoing basis to maintain localization of pre-synaptic complexes suggests a novel role for UNC-5 in maintaining the polarized localization of the pre-synaptic complexes in a manner independent of axon guidance or initial synaptic polarization. This is an intriguing finding and one that deserves investigation for other neurons and axon guidance molecules.

Two Wnt cues also control synapse localization in the DA9 neuron but in different regions than UNC-6/Netrin.16 LIN-44/Wnt emanating from the tail region (light pink patch in Fig. 2) causes exclusion of synapses from the more posterior section of the DA9 axon located in the dorsal cord (zone 4 in Fig. 2). A second Wnt, EGL-20 is also produced by tail cells (darker pink region in Fig. 2), and it excludes synapses from the region of the axon in the ventral cord (zone 2 in Fig. 2). Both Wnts cooperate to exclude synapses from zone 3. There is a strict correlation between the presence of the LIN-44/Wnt receptor, LIN-17/Fz, in zones 2, 3 and 4 and the absence of synapses in these regions. LIN-17/Fz is required cell-autonomously in DA9 to rescue synaptic localization defects. In the absence of LIN-44/Wnt, both the receptor LIN-17/Fz and the pre-synaptic complexes were mis-localized since they were now found in both zone 4 and 5 of the axon. Therefore, LIN-44/Wnt is instructive in regulating the location of the synapses in the axon of the DA9 neuron. Both LIN-44/Wnt and EGL-20/Wnt normally work cooperatively to exclude synapses, since animals lacking both had synapses in zones 3, 4 and 5 of DA9.

UNC-6/Netrin cooperates with the Wnt family members to exclude synapses from particular regions of the DA9 axon and only allow them to occur in regions free of the influence of both UNC-6/Netrin and the Wnts. Ectopic expression of UNC-6/Netrin and LIN-44/Wnt in various cells and genetic backgrounds was used to show that UNC-6/Netrin and LIN-44/Wnt could function interchangeably in excluding synapses in the DA9 neuron.14 Ectopic expression of UNC-6 in a posterior to anterior gradient close to DA9 caused RAB-3 to be localized more posteriorly in zone 5, rather than in both zone 4 and 5. The mis-localization was UNC-5 dependent and was seen regardless of whether or not DA9 was misguided. Ectopic UNC-6 could also rescue mis-localization defects in the absence of either
LIN-44/Wnt or its receptor LIN-17/Frizzled. These observations suggest that UNC-6/Netrin and LIN-44/Wnt both exclude synapses and can function together to control both axon guidance and pre-synaptic complex localization. Therefore, EGL-20/Wnt and LIN-44/Wnt work cooperatively with the UNC-6/Netrin ligand to inhibit the assembly of pre-synaptic complexes from inappropriate neuronal compartments. Synapses are excluded from the dendrite (zone 1) by UNC-6/Netrin, the region of the axon proximal to the cell body (zone 2) by EGL-20/Wnt, the commissures (zone 3) by EGL-44/Wnt and EGL-20/Wnt, and the distal portion of the axon (zone 4) by LIN-44/Wnt.14

It remains to be seen whether UNC-6/Netrin and its receptors are usually involved in synapse localization in C. elegans itself and in other organisms, beyond the highly specific cell contexts discussed. The involvement of molecules in both axon guidance and synaptogenesis is likely to be a general phenomenon, as the Netrins are expressed in the adult nervous systems of vertebrates including neurons and oligodendrocytes in the adult rat.17 DCC is expressed in the adult rat forebrain.18 UNC-5 is expressed in the heart and brain of adult vertebrates.19 Ephrins have also been shown to be involved in both axon guidance and synapse formation.20 Wnts have been found to play roles in regulating neuronal connectivity by controlling axon pathfinding, axon remodelling, dendrite morphogenesis and synapse formation in invertebrates and mammals.21 Recently, it was shown that pro- and anti-synaptogenic effects of Wnt proteins are associated with the activation of canonical and non-canonical Wnt signaling pathways in Drosophila and mouse.22,23 It is anticipated that many more instances of axon guidance molecules involved in synapse formation will be described. For instance, in the case of the synapse between the AIY and the RIA interneurons just discussed, AIY also synapses with two other interneurons, the AIZ and RIB but these synapses are not altered significantly in the absence of UNC-40/DCC/Fra. Presumably, these synapses require other molecules to guide synapse formation. Although the two receptors UNC-40 and UNC-5 are functioning with their normal ligand UNC-6/Netrin, it is not clear whether the remainder of the signaling pathways are conserved, and this question will be an interesting topic for future work on synapse formation.

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