

RESEARCH HIGHLIGHT

ENU-3 is a new player in UNC-6/Netrin directed axon outgrowth and guidance in *C. elegans*

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UNC-6/Netrin is an axon guidance cue that works through UNC-40/DCC/Frazzled to attract cells and neurons and through UNC-5 and UNC-40 to repulse cells and neurons during nervous system development. The gene encoding the novel protein ENU-3 was identified in a mutant that enhanced the axon outgrowth defects of the DA and DB classes of motor neurons in a strain lacking functional UNC-5. Mutations in ENU-3 also enhanced the axon outgrowth and guidance defects of the ventrally directed processes of the AVM and PVM touch receptor neurons in a strain lacking functional UNC-40. It also plays a role in adhesion of axons to the substratum as does UNC-40. The ENU-3 protein is a putative single pass transmembrane protein expressed throughout the nervous system whose possible biochemical roles are currently unknown.

Keywords: UNC-6/Netrin; UNC-40/DCC; ENU-3; axon outgrowth; guidance; *C. elegans*

Abbreviations: anterior ventral microtubule neuron, AVM; cell death, CED ; dorsal nerve cord, DNC; egg laying defective, EGL; enhancer of Unc, ENU; hermaphrodite specific neuron, HSN; migration defective, MIG; posterior lateral microtubule neuron, PLM; posterior ventral microtubule neuron, PVM; Rap1-GTP-interacting adapter molecule, RIAM; uncoordinated, UNC; ventral nerve cord, VNC

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UNC-6/Netrin and its receptors

Development of the nervous system is a highly stereotyped process usually resulting in a perfectly patterned organism with synapses in the correct places. The process of establishing the correct architecture of the nervous system is guided by cues including the Netrins and Slits that result in changes in the cytoskeleton of the growth cones at the tips of the developing, migrating axons resulting in guided movements ^[1,2].

The laminin-like guidance cue UNC-6/Netrin is a

bifunctional molecule that guides the migrations of cells and axons during development of the nervous system in *C. elegans* ^[3,4]. Mutants have guidance defects in many neurons resulting in a severe uncoordinated phenotype in the absence of UNC-6. UNC-6 is expressed dynamically in glia and neurons that serve as guidepost cells ^[5]. The vertebrate UNC-6 homologues are the Netrins ^[6,7,8], reviewed in reference [9]. UNC-5 and UNC-40/DCC are two of the UNC-6 receptors ^[10, 11]. DSCAM (Downs Syndrome Cell Adhesion Molecule) has also been discovered to be a Netrin receptor but it has not been

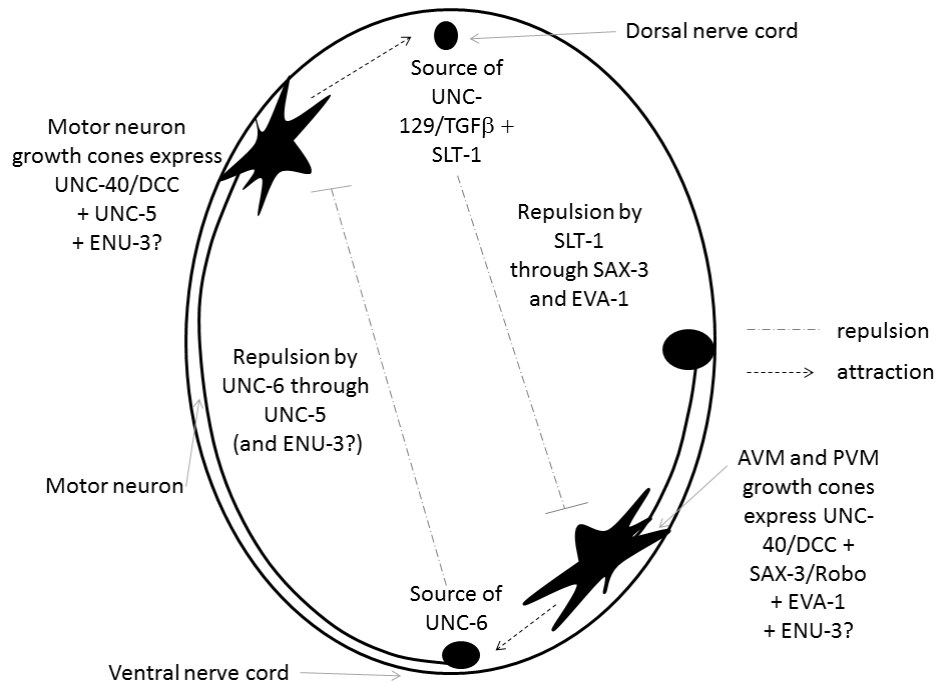


Figure 1. Netrin and SLT-1 sources in *C. elegans* and their effects on axon guidance. The DA and DB motor neurons on the left express the UNC-40 and UNC-5 receptors and are repulsed from their cell bodies in the ventral cord by UNC-6 [4,5,10,11]. The outgrowth of their axons from their cell bodies is influenced by ENU-3 [23,25]. They are also influenced by dorsal sources of UNC-40:UNC-5 heterodimers, more favoured for long-range migrations [22]. The AVMs and PVMs on the right side of the diagram express UNC-40 and ENU-3 and are attracted towards UNC-6 [11,23]. They also express SAX-3/Robo and EVA-1 and are repulsed by dorsal sources of SLT-1 [28,29,30].

described in *C. elegans* [12,13,14]. A schematic of the locations of the cues and receptors in the developing nervous system of *C. elegans* is shown in Figure 1.

Cells and axons that migrate towards ventral sources of UNC-6 in *C. elegans* express UNC-40/DCC/Frazzled (Deleted in Colorectal Cancer) suggesting an attraction of UNC-6 for UNC-40 [11]. DCC is a Netrin receptor that is expressed by spinal commissural axons that migrate towards the floor plate of the spinal cord [15]. Cells and axons that migrate away from sources of UNC-6 in *C. elegans* express both UNC-5 and UNC-40 [10,11,16,17,18]. Vertebrate UNC5H1 (also known as RCM Rostral Cerebellar Malformation) is expressed by the motor neurons that migrate away from the floor plate of the vertebrate spinal cord [19, 20]. Vertebrate UNC5H1 was shown to bind to Netrin/UNC-6 [19]. The importance of the intracellular regions of the receptors was demonstrated when the ligand-gated interaction between the cytoplasmic domains of UNC5H1 or UNC5H2 and DCC was triggered by Netrin 1 and caused switching of the response of *Xenopus* spinal neurons from attraction to repulsion [21].

The main molecule causing steering towards sources of UNC-6 is likely to be an UNC-40 homodimer while the main molecule causing steering away from sources of UNC-6 is likely to be UNC-5. Somewhat surprisingly, UNC-5 homo-dimers are dissociated in favour of UNC-40:UNC-5 heterodimers due to the action of the TGFβ - related UNC-129 to ensure correct long-range migrations of motor neurons away from ventral sources of UNC-6 [22].

ENU-3 enhances the motor neuron axon outgrowth and guidance defects of a strain lacking UNC-5

In *C. elegans* lacking either UNC-5 or its cue UNC-6/Netrin, the motor neurons are misguided such that they rarely, if ever, reach the dorsal cord [3, 10]. However, most of the axons successfully exit the ventral nerve cord (VNC), suggesting the existence of an additional pathway or mechanism that ensures motor neuron axon outgrowth. In order to identify axon outgrowth proteins, a genetic enhancer screen was conducted for increased DA and DB motor neuron axon outgrowth defects in a strain lacking functional UNC-5 [23]. One of the strains found in the screen was mutant for ENU-3 (Enhancer of Unc). A

mutation in the gene that encodes ENU-3 enhanced the axon outgrowth defects of a subset of the motor neurons in a strain lacking functional UNC-5. Somewhat to our surprise, the mutation also enhanced the axon outgrowth defects of more limited number of neurons in a strain lacking functional UNC-6. These data suggested that the ENU-3 protein functions in a pathway parallel to UNC-6 for motor neuron outgrowth. However, that conclusion became more dubious when we discovered that a second strain with an alteration in UNC-6 had more severe axon outgrowth defects than a strain lacking functional UNC-6. This mutant, *unc-6(e78)*, expresses a version of UNC-6 with a missense mutation in the V-3 domain that allows the protein to interact with UNC-40 but not UNC-5^[24]. However, the axon outgrowth defects of this second mutant were also enhanced somewhat by lack of ENU-3 as was the strain lacking UNC-6, still supporting the idea that ENU-3 works in a pathway independent of UNC-6 for motor axon outgrowth^[25].

The unexpected observation that mutants lacking both UNC-40 and the version of UNC-6 encoded by *unc-6(e78)* had almost no axon outgrowth defects led us to the hypothesis that if UNC-40 can bind to UNC-6, it can oppose outgrowth of the motor neurons^[25]. The idea that UNC-40 can oppose axon outgrowth in the absence of UNC-5 was tested by the construction of an *unc-5:unc-40* double null strain which had very few motor neuron outgrowth defects. Therefore, in the absence of UNC-5, UNC-40 opposes axon outgrowth, probably because the UNC-40 homodimers are attracted towards ventral sources of UNC-6. This observation is consistent with the observation by Lundquist's group that the presence of UNC-40 increased the guidance defects in the VD and DD motor neuron axons in the absence of UNC-5^[26]. One possibility for a role for ENU-3 is that it favours the formation of UNC-5:UNC-40 heterodimers rather than UNC-40 homodimers, as they guide long-range migrations of motor neurons in both *Drosophila* and *C. elegans*^[22,27]. However, ENU-3 is also likely to have an additional UNC-6 independent role as a strain lacking UNC-40, ENU-3 and UNC-5 had more DA and DB axon outgrowth defects than one lacking UNC-40 and UNC-5 but less than one lacking UNC-5 and ENU-3^[25]. We are currently trying to further understand the role of ENU-3 in motor neuron outgrowth.

ENU-3 is predicted to be a novel putative transmembrane protein of 204 amino acids that is expressed throughout the nervous system, particularly in the cell bodies^[23]. It has a predicted signal peptide of 21 amino acids, a putative extracellular region followed by a predicted transmembrane domain from amino acids 109-

128. After the transmembrane domain there are two regions of low complexity (from amino acids 151 to 165 and 181 to 191) that are likely to be intracellular. ENU-3 has five paralogs in *C. elegans*, all novel proteins that are longer than ENU-3. Currently, we are continuing to determine the roles of all six of the ENU-3 family of proteins and their relationship with UNC-6, and its two receptors UNC-5, UNC-40 in motor neuron outgrowth.

ENU-3 works parallel to UNC-40/DCC downstream of UNC-6/Netrin in guidance of the touch receptor neurons

More recently, we turned our attention to the question of whether ENU-3 plays a role in guidance of the processes of the touch receptor neurons AVM and PVM in the ventral direction, towards sources of UNC-6. These processes are guided by attraction towards ventral sources of UNC-6 working through UNC-40^[11] and repulsion by dorsal sources of SLT-1/Slit^[28] working through SAX-3/Robo^[29] and EVA-1^[30]. The first question was whether an *enu-3* mutant had AVM or PVM guidance defects and it did not^[25]. We then constructed a double mutant strain lacking both ENU-3 and UNC-40 and found to our surprise that there were more AVM and PVM defects in the strain lacking both UNC-40 and ENU-3 than those lacking UNC-40 alone. In fact, the extent of the defects in the absence of both UNC-40 and ENU-3 was approximately the same as in the strain lacking functional UNC-6. A mutation in *enu-3* did not enhance the ventral guidance defects of a strain lacking UNC-6 but did enhance a strain lacking functional SLT-1 confirming that ENU-3 works in the UNC-6 pathway. These results put ENU-3 in the UNC-6 pathway working parallel to UNC-40 for the guidance of AVM and PVM ventrally directed processes.

It appears as if ENU-3 also plays a role in adhesion of axons to the substratum. DCC/UNC-40 has been described as having an attachment function^[31] but this is not usually apparent in *unc-40* mutant animals^[25]. *enu-3* mutants had mild misguidance defects in some of the DA and DB motor neurons but they looked as if they were attached properly^[23]. The AVM and PVMs looked normal in an *enu-3* mutant^[25]. However, the strain lacking both UNC-40 and ENU-3 had defects in the attachment of some of the touch receptor neurons, particularly of the PLMs whose processes migrate in the posterior to anterior direction towards the head. The attachment defects indicate that both UNC-40 and ENU-3 proteins have a role in the architecture of the nervous system.

Redundancy of the UNC-6 and the SLT-1 signaling

pathways.

Our data indicate that ENU-3 has a role in axon outgrowth and guidance of the dorsally directed motor neurons in a pathway parallel to UNC-6 as lack of ENU-3 affects motor neuron outgrowth in the absence of UNC-5 or UNC-6 [23]. However, ENU-3 works in an UNC-6 dependent pathway in guidance of the ventrally directed processes of the AVMs and PVMs [25]. The true role of ENU-3 is difficult to assign since it appears to be both UNC-6 dependent and independent. Perhaps it is a molecule shared by two pathways in promoting axon outgrowth of the motor neurons, one being UNC-6 and a second unknown pathway with a receptor besides UNC-40. In some cases there is considerable redundancy in signaling as for instance where both UNC-6 and SLT-1 work at least partly through a common pathway in guidance of AVM and PVM. There are at least two pathways that work in the AVM and PVM. In one pathway CED-10/RacGTPase and UNC-115/AbLIM work may together and in parallel to another pathway containing UNC-34/enabled/VASP downstream of UNC-6 [32] or both UNC-6 and SLT-1 [33]. A mutation affecting the cytoplasmic adaptor protein MIG-10/RIAM/Lamellipodin enhanced the AVM and PVM defects of a strain lacking either UNC-6 or SLT-1/Slit [34]. Over-expression of MIG-10 in the absence of UNC-6 and SLT-1 induced a multipolar phenotype that was rescued to a unipolar morphology by either UNC-6 or SLT-1, suggesting that they can work through a common pathway.

Protein localization and axon outgrowth

Asymmetric protein localization has been demonstrated in some cases in *C. elegans* nervous system development. The hermaphrodite specific neurons (HSN) make ventrally directed migrations guided by UNC-6 but not SLT-1. In this process, UNC-6 causes polarization of the UNC-40 receptor followed by a sharp and distinct localization of MIG-10/RIAM/Lamellipodin on the ventral side of the HSN cell body prior to neurite outgrowth [35]. MIG-10 is localized by CED-10/Rac and it was suggested that they function together to cause cytoskeletal assembly resulting in polarized outgrowth controlled by UNC-6, presumably through its receptor UNC-40 [36]. The asymmetric protein localization of MIG-10 in the HSN cell body is presumed to lead to polarization of the growth cone of the neurite that will go on to form a growth cone of an axon that exits and migrates towards ventral sources of UNC-6.

UNC-6/Netrin promotes a balance between enhancement and inhibition of protrusion of growth cones that results in axon guidance. The cellular role of ENU-3

in growth cone outgrowth is not understood and it is not entirely clear where it fits with UNC-6 and its receptors. A study that looked at growth cone dynamics showed that lack of functional UNC-5 resulted in increased protrusion of growth cones in the VD and DD motor neurons while lack of either UNC-40 or UNC-6 resulted in decreased protrusion of the growth cones in the same neurons [26]. Lack of UNC-5 also caused an increase in the area of the growth cones and the duration of the filopodia. In the absence of UNC-5, abnormally long, stable filopodia were observed but only when UNC-40 and UNC-6 could interact, suggesting that the interaction of UNC-40 with UNC-6 was responsible for the unusual filopodia. The conclusion was that UNC-5 normally limits the protrusion of growth cones that are promoted by the interaction of UNC-40 and UNC-6. This study found evidence for independence of polarity of growth cone protrusion and filopodial dynamics which are both factors involved in growth cone guidance.

A more complicated role for UNC-5 than a simple chemorepulsion by UNC-6 was suggested by the observation that the absence of both the UNC-5 receptor and the cytoskeletal binding protein UNC-53/NAV2 caused randomized axon outgrowth and distribution of UNC-40::GFP in the HSN neurons similar that that seen in the absence of UNC-6 [37]. They suggested that UNC-53 and UNC-5 work in parallel pathways to control the localization of UNC-40 in an UNC-6 dependent fashion and that the localization of UNC-40 results in leading edge formation. This was surprising as UNC-5 was not known to be involved in ventrally directed guidance. Mutations in the genes that encode EGL-20/WNT and Vangl also affected localization of UNC-40 in the HSNs. Since axons that migrate in the anterior-posterior directions are affected by the Wnts, perhaps localization of axon outgrowth in these neurons is also triggered by UNC-40 localization. Possibly, ENU-3 plays a role in localization of another unknown UNC-6 dependent receptor. Further cell biology experiments will cast further light on the true role of ENU-3.

It is probable that similar work to that described for UNC-40 will be described for other receptors as there are clearly many other receptors involved in axon outgrowth processes besides UNC-40 including the two SLT-1 receptors SAX-3/Robo and EVA-1 [28,29]. In the case of the AVMs and PVMs, one of the receptors involved in guidance is likely to be UNC-6 dependent since the lack of UNC-40 results in less axon outgrowth defects than the lack of UNC-6. Our data indicate that ENU-3 is likely to be in this second UNC-6 dependent pathway for AVM and

PVM guidance. The DA and DB motor neurons usually exit the ventral cord correctly in the absence of functional UNC-5 and UNC-40 receptors as well as in the absence of functional UNC-6. This suggests that there is likely to be another UNC-6 independent pathway involved in outgrowth of these motor neurons which is what prompted our search for additional axon outgrowth proteins. This second pathway includes ENU-3 and appears to involve a receptor besides UNC-5 and UNC-40 that is required for motor neuron axon outgrowth. Further work on the biochemical activity of ENU-3 should lead to a deeper understanding of axon outgrowth proteins and mechanisms.

Netrin, UNC-5 and DCC have been found to play a role in guidance of many neurons throughout the vertebrate nervous system as well as the development of the cardiovascular, lung and breast tissue so their roles appear to be widespread in development (reviewed in [9,38]). Abnormalities of expression of all three have also been implicated in several types of cancer including colon cancer where UNC5 and DCC are considered to be dependence receptors (reviewed in [39, 40, 41]), so further understanding of their roles in nervous system development may have clinical implications for problems beyond the nervous system. Continued research on the Netrin signaling pathway will provide exciting prospects for translational medicine and therapeutic interventions in cancer treatment.

Conflicting interests

The authors have declared that no competing interests exist.

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