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The ENU-3 protein family members function in the Wnt pathway parallel to UNC-6/Netrin to promote motor neuron axon outgrowth in *C. elegans*

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ABSTRACT

The axons of the DA and DB classes of motor neurons fail to reach the dorsal cord in the absence of the guidance cue UNC-6/Netrin or its receptor UNC-5 in *C. elegans*. However, the axonal processes usually exit their cell bodies in the ventral cord in the absence of both molecules. Strains lacking functional versions of UNC-6 or UNC-5 have a low level of DA and DB motor neuron axon outgrowth defects. We found that mutations in the genes for all six of the ENU-3 proteins function to enhance the outgrowth defects of the DA and DB axons in strains lacking either UNC-6 or UNC-5. A mutation in the gene for the MIG-14/Wntless protein also enhances defects in a strain lacking either UNC-5 or UNC-6, suggesting that the ENU-3 and Wnt pathways function parallel to the Netrin pathway in directing motor neuron axon outgrowth. Our evidence suggests that the ENU-3 proteins are novel members of the Wnt pathway in nematodes. Five of the six members of the ENU-3 family are predicted to be single-pass trans-membrane proteins. The expression pattern of ENU-3.1 was consistent with plasma membrane localization. One family member, ENU-3.6, lacks the predicted signal peptide and the membrane-spanning domain. In HeLa cells ENU-3.6 had a cytoplasmic localization and caused actin dependent processes to appear. We conclude that the ENU-3 family proteins function in a pathway parallel to the UNC-6/Netrin pathway for motor neuron axon outgrowth, most likely in the Wnt pathway.

1. Introduction

During development, neuron cell bodies send out many neurites with growth cones at the tips (Farrar and Spencer, 2008; Tamariz and Varela-Echavarría, 2015). A single neurite is chosen as an axon and migrates to the final destination due to directed alterations that occur in the actin cytoskeleton (Heckman and Plummer, 2013). The axon may migrate over long distances before synapsing with its final partner, allowing communication between adjacent cells. The whole highly stereotyped process of axon migration involves expression of guidance cues at the appropriate times and locations that guide the growth cones of neurons expressing the corresponding receptors (Vitriol and Zheng, 2012; Kolodkin and Tessier-Lavigne, 2011; Killeen and Sybingco, 2008). The main guidance cues described to date include the Netrins, Wnts, Slits, Semaphorins and Ephrins, and in addition some morphogenic proteins can also function as guidance cues, such as Shh and the TGFβ family (Charron and Tessier-Lavigne, 2007; Kolodkin and Tessier-Lavigne, 2011; Yam and Charron, 2013). In guidance of neurons, the cues can be attractive or repulsive, depending on the receptors present. This is important, as once axons enter a region such as the ventral midline they may need to be able to dissociate from previously attractive molecules to continue migrating towards their final destinations. Furthermore, a single axon may be subject to opposing cues simultaneously, so decisions need to be made within a developing growth cone regarding axon outgrowth and guidance. Since axon guidance defects appear to be relatively rare, the guidance mechanisms must be both robust and probably somewhat redundant.

In the developing nervous system of *C. elegans* the motor neurons are guided by the cue UNC-6 (uncoordinated), that is expressed in various places along the ventral nerve cord including glia and guidepost cells (Ishii et al., 1992; Wadsworth et al., 1996). The receptors in motor neurons include UNC-5 and UNC-40 (Leung-Hagesteijn et al., 1992; Chan et al., 1996). In the absence of UNC-6 or the UNC-5 receptor, the axons of the DA and DB classes of motor neurons usually exit their cell bodies in the ventral nerve cord but fail to reach the dorsal nerve cord (Hedgecock et al., 1990). The presence of UNC-40 is less critical to guidance of the DA and DB motor neuron axons since they sometimes reach the dorsal nerve cord in its absence.

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Nevertheless, UNC-40 is needed for the effective long-range migrations of the motor neuron axons and it has been suggested that in low UNC-6 concentrations, UNC-40:UNC-5 heterodimers provide more robust axon guidance than UNC-5 homodimers (MacNeil et al., 2009). Besides the UNC-6 pathway, other pathways that control motor neuron axon outgrowth have not been described in *C. elegans*.

In *C. elegans*, the distal tip cell uses some of the same guidance pathways as the motor neurons (Antebi et al., 1997). The distal tip cells are at the leading edge of the developing gonad and migrate from beside the vulva along the ventral side of the body, turn and migrate towards the dorsal cord where they reflex back along the dorsal side towards mid-body. The UNC-6 pathway is involved in guiding the migrations of both the distal tip cells and the motor neuron axons. The dorsal migrations of the distal tip cells are co-incident with the expression of UNC-5 and the timing of the migrations can be altered by premature or delayed expression of UNC-5 (Su et al., 2000; Killeen et al., 2002).

The UNC-6 guidance pathway is conserved in higher organisms. The mammalian homologues of UNC-6 are called the Netrins (Serafini et al., 1994, 1996; Kennedy et al., 1994). There are four Netrin receptors: (i) DCC (deleted in colorectal cancer), an UNC-40 homologue (Keino-Masu et al., 1996), (ii) Neogenin, a second DCC/UNC-40 homologue (Chan et al., 1996), (iii) DSCAM, the Downs Syndrome cell adhesion molecule (Ly et al., 2008) and (iv) Unc-5 (Leonardo et al., 1997; Ackerman et al., 1997). DCC expression causes attraction towards ventral sources of Netrin but UNC-5 expression overcomes the chemo-attraction of Netrin for DCC, allowing the trochlear motor neurons to grow away from sources of Netrin (Hong et al., 1999).

The observation that the DA and DB classes of motor neurons usually exit the ventral nerve cord, even in the absence of UNC-6/Netrin or UNC-5, while the VD and DD classes of motor neurons usually fail to exit in the absence of UNC-5, led to the idea that there could be another pathway besides the UNC-6 pathway that is also responsible for their outgrowth. A screen to find motor neuron axon outgrowth mutants in an UNC-5 null mutant background was conducted and we found that a newly identified protein called ENU-3.1 (Enhancer of Unc) was involved (Yee et al., 2011). Mutations in the *enu-3.1* gene enhanced the low number of DA and DB motor axon outgrowth defects observed in strains lacking functional versions of either UNC-5 or UNC-6. ENU-3.1 is expressed ubiquitously throughout the nervous system and functions cell autonomously when expressed in the affected neurons (Yee et al., 2011).

Based on searches for genes and proteins of homologous sequences we found that ENU-3.1 has five paralogues in the *C. elegans* genome, all of previously unknown function. The current study was aimed mainly at finding whether all six of the members of the ENU-3 protein family in *C. elegans* function to promote outgrowth of the axons of the DA and DB motor neurons in the absence of UNC-5. Although many of the pathways that control axon outgrowth and guidance are known, there is much to be uncovered about how each of these is regulated. ENU-3 proteins may thus function to coordinate signaling in one, some, or all of the known outgrowth/guidance signaling pathways, which will be examined further here. We wished to determine the subcellular localization of the proteins to provide clues to function.

2. Materials and methods

2.1. Strains and handling

Strains were handled using standard procedures and were grown at 20 °C unless indicated otherwise (Brenner, 1974; Hope, 1999). The background strain used was N2, which is usually regarded as the wild-type strain. In addition the following alleles were used: LGI: *unc-40(e1430); enu-3.3(tm5189)* (W03G9.3); *enu-3.4(tm6465)* (W05F2.2); LGII: *mig-14(ga62), mig-14(k124);* LGIII: *enu-3.1(tm4519)* (H04D03.1); *enu-3.2(tm5039)* (C38D4.1); *enu-3.5(tm5256)* (Y37D8A.12); *enu-3.6(tm3797)* (KO1G5.3); LGIV: *unc-5(e53); evIs82b [unc-129p::gfp;dpy-20],* LGV: *him-5(e1490);* LGX: *unc-6(ev400)*. The *tm* strains were isolated by and obtained with gratitude from the Mitani group.

2.2. Axon outgrowth

Motor axon outgrowth was assessed as described in Yee et al. (2011) using the *evIs82b* transgene that carries the *unc-129* neuronal promoter driving green fluorescent protein (GFP)(Colavita et al., 1998). Briefly, animals were mounted on agarose pads in levamisole and examined under epi-fluorescence on a compound, upright Leica DM5000B microscope under magnification of 200X, 400X or sometimes 630X. Cell bodies without detectable axons exiting the ventral nerve cord were scored as motor neuron axon outgrowth defective.

2.3. RNA interference

Animals from various strains indicated in the caption for the appropriate Figure and Table were grown on bacterial clones expressing double stranded RNA corresponding to the *C. elegans* gene of interest (Kamath et al., 2003). The experiments were conducted as described (Kamath et al., 2003) with modifications used by Yee et al. (2011) and 2014. Motor neuron axon outgrowth defects were scored in F2 animals at the L4 stage of development. Each experiment was performed three times and 100 animals were counted for each trial. The controls included a no treatment sample and a sample treated with the vector lacking an insert (pL4440). Statistical significance was evaluated using χ^2 or *t*-test analysis.

2.4. Expression of proteins in C. elegans

A first PCR product containing 3 kb of the *enu-3.1* promoter and the entire gene was made from genomic DNA using elongase polymerase (Thermo Fisher Scientific). A second PCR product containing the *gfp* gene and the *unc-54* 3'UTR was made from pPD95.75. A third PCR product was made containing a modified *unc-86* promoter containing two copies of the core promoter sequence (a gift from Richard Ikegami and Kazuko Fujisawa). A fusion product of ENU-3.1::GFP was made by PCR using the first and second reaction products (Hobart 2002). ENU-3.1::GFP driven by the *unc-86* promoter was then made by PCR from the first fusion gene product and the third PCR product. The final fusion product was microinjected at approximately 30 ng per microliter into the gonads of N2 animals along with 2 ng per microliter pCFJ90 (plasmid carrying *myo-2::mcherry::unc-54* UTR (a gift from Erik Jorgensen). Animals that were transgenic for the *myo-2::mcherry* were examined by confocal microscopy.

2.5. Expression of proteins in mammalian cells

Standard molecular biology procedures were followed (Sambrook and Russell, 2006). A construct for expression of ENU-3.1 in mammalian cells was made as follows: the *enu-3.1* (H04D03.1) cDNA was purchased from Thermo Scientific and was cloned in its entirety by "seamless cloning" into the mammalian expression vector pCDNA3 (Lu, 2005; Invitrogen). The cDNA was made from the original clone using PCR products designed to allow homologous recombination of the products. The vector containing the *enu-3.1* cDNA was subjected to a second round of seamless cloning to insert a FLAG tag following the predicted signal peptide after amino acid 24 using the primers 5' GGT-GGT-TGT-TGG-TAA-TAC-ATG-AAC-TAT-TAA-TCC-GAA-G 3' and 5' A-CCA-ACA-ACC-ACC-GAT-TAC-AAG-GAT-GAC-GAC-GAT-AAG-ACT-ACT-ACG-ACA-CCA-AAT-GTG 3'. The signal peptide is predicted to be cleaved after amino acid 21. The primers restore four threonines after the FLAG domain so that the final predicted protein starts from amino acid 21. The protein made from this construct was called