Expression of the *unc-4* homeoprotein in *Caenorhabditis elegans* motor neurons specifies presynaptic input

David M. Miller III* and Charles J. Niemeyer
Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

*Author for correspondence

SUMMARY

In the nematode, *Caenorhabditis elegans*, VA and VB motor neurons arise from a common precursor cell but adopt different morphologies and synapse with separate sets of interneurons in the ventral nerve cord. A mutation that inactivates the *unc-4* homeodomain gene causes VA motor neurons to assume the VB pattern of synaptic input while retaining normal axonal polarity and output; the disconnection of VA motor neurons from their usual presynaptic partners blocks backward locomotion. We show that expression of a functional *unc-4*-β-galactosidase chimeric protein in VA motor neurons restores wild-type movement to an *unc-4* mutant. We propose that *unc-4* controls a differentiated characteristic of the VA motor neurons that distinguishes them from their VB sisters, thus dictating recognition by the appropriate interneurons. Our results show that synaptic choice can be controlled at the level of transcription in the post-synaptic neuron and identify a homeoprotein that defines a subset of cell-specific traits required for this choice.

Key words: *unc-4*, neural specificity, homeoprotein, *Caenorhabditis elegans*, motor neurons

INTRODUCTION

The generation of complex neural tissues depends on the capacity of neurons to adopt traits that distinguish them from their neighbors. Important differences include the trajectory of axonal outgrowth and the specificity of synaptic connections which together define the structure and function of neural circuits. A major goal of developmental biology is to link neuronal differentiation to the creation of these specific networks.

In the nematode, *Caenorhabditis elegans*, most of the VA and VB motor neurons arise from a common precursor cell (Sulston and Horvitz, 1977). However, they adopt distinctive morphologies and accept different synaptic inputs in the ventral nerve cord (Fig. 1A) (White et al., 1986); VA motor neurons extend anteriorly directed axons and are connected to interneurons that mediate backward movement while VB motor neurons have posteriorly directed axons and synapse with a separate group of interneurons that mediate forward movement (Chalfie et al., 1985). A null mutation in the *unc-4* gene results in the disconnection of VA motor neurons from their usual presynaptic partners, thereby blocking reverse locomotion. The VA motor neurons instead receive input from an interneuron that normally synapses with the VB motor neurons. These changes in connectivity are not accompanied by any visible effects on process placement or axonal morphology in the ventral nerve cord (Fig. 1B). Thus, the *unc-4* mutation causes VA motor neurons to assume the pattern of synaptic input of their VB sister cells while retaining normal axonal polarity and output (White et al., 1992).

We have previously shown that *unc-4* encodes a homeodomain protein (Miller et al., 1992). The fact that VA and VB arise from the same parent cell is consistent with a model in which the *unc-4* homeoprotein is required to distinguish the fates of these sister cells with respect to synaptic input. Alternatively, *unc-4* could govern the differentiation of the interneurons that provide input to the VA and VB motor neurons. In order to distinguish between these models and to identify the cell type in which *unc-4* acts, we have generated a transgenic nematode line in which a functional *unc-4*-β-galactosidase chimeric protein is expressed under the control of the *unc-4* promoter region. We demonstrate that the restoration of backward movement to an *unc-4* mutant depends on the expression of the *unc-4*(+):lacZ transgene in the VA motor neurons. No X-gal staining is detected in interneurons or in VB motor neurons in these animals. We therefore propose that *unc-4* controls, at the level of transcription, some feature of the VA motor neurons that distinguishes them from their VB sisters and thereby dictates the choice of presynaptic partners. A preliminary account of this work is described in Miller et al. (1993).

MATERIALS AND METHODS

Nematode strains

The strain, *wdIs1*, is a chromosomal integrant of the *unc-4*-lacZ plasmid, pNC4-22Lz (Fig. 2). Other nematode strains used in this study (Brenner, 1974) were obtained from the Caenorhabditis Genetics
Molecular biology and construction of lacZ plasmids

The 5.5 kb PstI-KpnI fragment from the unc-4 genomic region of cosmid C07E2 was cloned into Bluescript to create pNC4-21. To construct pNC4-22Lz, the 3.2 kb PstI-SmaI fragment from pNC4-21 was subcloned into PstI-SmaI-digested pPD21.28 lacZ expression vector (Fire et al., 1990). To construct the unc-4(+)/lacZ expression vector, pNC4-23Lz, unc-4 cDNA was obtained by reverse transcription PCR (Kawasaki, 1990) using primers u4p9 to the first exon on the left in Fig. 2 and u4p10 which is complementary to unc-4 coding sequence near the 3' terminus of the translated region (last exon on the right in Fig. 2) and includes an adaptor with a BamHI site (Miller et al., unpublished data). A 580 bp SmaI-BamHI cDNA fragment derived from the u4p9/u4p10 PCR product was ligated with the 3.2 kb PstI-SmaI genomic fragment from the unc-4 upstream region and PstI-BamHI cut pPD21.28 lacZ. pNC4-23Lz encodes the complete unc-4 protein with the exception of five amino acids at the carboxy terminus. To create pNC4-76Lz, the 2.8 kb HindIII-SmaI fragment from the unc-4 upstream region (Fig. 2) was ligated into HindIII-SmaI cut plasmid p76-L18, which contains a 0.6 kb unc-76 C DNA fragment fused in the correct translational reading frame with lacZ in the expression plasmid pPD21.28 [L. Bloom and H.R. Horvitz, personal communication]. The presence of the unc-76 domain excludes X-gal staining from the cell nucleus (Miller et al., 1993) but recent results indicate that the removal of the unc-76 segment from pNC4-76Lz has no detectable effect on the intensity of axonal staining (data not shown).

Transformation/rescue

For all transformation experiments, DNA was injected into the syncytial gonad of adult hermaphrodites (Mello et al., 1992). A wild-type copy of the dpy-20 gene (pMH86) (D. Clark and D. Baillie, personal communication) was used as a cotransformation marker to complement the mutation dpy-20 (e1282) in the recipient strain; transformed animals exhibit a wild-type body shape (i.e. are non-Dpy). Due to the temperature sensitivity of the Dpy-20 phenotype, animals were cultured at 25°C.

dpy-20(e1282); unc-4(wd1) hermaphrodites were injected with a solution of tester DNA (10-100 µg/ml), plasmid carrier DNA (pBluescript) + tester DNA = 100 µg/ml, and pMH86 at 30 µg/ml. F2 progeny were evaluated for cotransformation; tester DNA was scored as positive if a majority of the plates giving rise to non-Dpy F2 progeny were also non-Unc.

unc-4-lacZ transgenic lines were obtained by picking non-Dpy F2 progeny of dpy-20(e1282) hermaphrodites injected with unc-4-lacZ expression plasmid and pMH86 as above. The presence of an unc-4-lacZ transgene was confirmed by staining with X-gal (see below). A chromosomal integrant (wdls1) of pNC4-22Lz, was obtained by X-irradiation (4000 Rad) of a transgenic line carrying pNC4-22Lz and pMH86 as an unstable extrachromosomal array (integration site <1 m.u. from unc-32 on III).

Animals were fixed (Finney and Ruvkun, 1990; Miller and Shakes, 1995) and incubated with X-gal solution to detect β-galactosidase-expressing cells and counterstained with DAPI (diamidino phenylindole) to reveal all cell nuclei (Fire et al., 1990). Alternatively, fixed
animals were stained with mouse monoclonal anti-β-galactosidase (1/100 dilution) (Promega) and rhodamine-coupled goat antimouse secondary antibody (1/100 dilution) (Sigma) preadsorbed with an acetone powder of wild-type *C. elegans* to reduce nonspecific staining (Miller and Shakes, 1995).

**Identification of X-gal stained cells**

Stained cells in unc-4(+)/lacZ and wdsI transgenic animals were identified on the basis of position relative to other cell nuclei visualized with DIC optics or DAPI fluorescence. X-gal staining was first observed about midway through embryogenesis (~400 minutes at 20°C) but cell identities in the embryo could not be reliably assigned until the 4 fold stage or in the L1 larva after hatching. The positions of motor neurons DA2-DA7 in the queue of L1 ventral cord motor neurons are invariant (fig. 14 in Sulston et al., 1983). Other X-gal stained neurons were identified by reference to published drawings: DA8 and DA9 (fig. 14d in Sulston et al., 1983) DA1, SABD, SABVL, SABVR (fig. 14b in Sulston et al., 1983); 15 (fig. 23c in Albertson and Thomson, 1976). Cell identities were confirmed by examination of axonal morphology (White et al., 1986) in the *pNC4-76Lz* transgenic animals stained with anti-β-galactosidase.

lacZ-positive cells arising in the first larval stage were typically scored in the L2. Identities were established from cell positions shown in published diagrams (fig. 5 in Sulston, 1976, fig. 14a in Sulston et al., 1983, fig. 16 in Sulston and Horvitz, 1977) and from axonal morphology (White et al., 1986) as revealed by staining a *pNC4-76Lz*-bearing strain with anti-β-galactosidase.

**Correlating unc-4(+)/lacZ expression with unc-4 rescue**

Non-Dpy wild-type larvae and Unc-4 larvae arising from the transgenic *unc-4(wd1)* animal carrying the *unc-4(+)/lacZ* extrachromosomal array were picked into two separate pools for X-gal staining. After incubating in X-gal solution for 24-48 hours at 37°C, the animals were pipetted onto a glass slide and evaluated using a 100× DIC (NA 1.25) objective in a Nikon Microphot microscope. Randomly selected lacZ-positive L2 larvae on each slide were scored until a total of 20 wild-type and 20 Unc-4 animals were evaluated.

**Reverse transcription-PCR to generate a developmental profile of unc-4 mRNA**

A synchronized population of the first larval stage (L1) was obtained by allowing embryos (Sulston and Hodgkin, 1988) to hatch overnight on unseeded NGM agar plates. Growth was initiated by placing the ‘starved’ L1s on NGM plates inoculated with bacteria (OP50). Plates were kept at 20°C. Groups of animals were collected at intervals to obtain samples of larval stages and adults. Developmental age was confirmed by direct observation of a sample from each population by Nomarski optics. Alternatively, worms were synchronized by allowing eggs to hatch on a 20 μm nylon screen (Spectrum) dipped in a pool of M9 buffer. L1 larvae that crawled through the screen into the M9 buffer in a 2-3 hour period were collected by centrifugation and then distributed to NGM-OP50 plates for growth. Harvested animals were kept at 30% sucrose, washed with 0.1 M Tris, pH 8, quick frozen in liquid nitrogen, and stored at –80°C for RNA isolation (Miller et al., 1992).

cDNA was synthesized from 2.5 μg of total RNA from each sample in a 20 μl reaction volume (Kawasaki, 1990). cDNA from 25 ng RNA was submitted to 20 PCR cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds) in the presence of 25 pmole of each of the *unc-4*-specific primers, u4p4 and u4p2, and 1 pmole of each of the *unc-4*-specific primers, u542 and u544, in a total volume of 50 μl. *unc-4* mRNA was simultaneously amplified in each reaction as an internal control. *unc-4* mRNA was maintained at a constant steady state level throughout *C. elegans* larval development (Honda and Epstein, 1990) and encodes a myosin heavy chain (Karn et al., 1983). A 370 bp PCR product was obtained from the *unc-4* primers u4p4 and u4p2 and a 615 bp fragment was derived from the *unc-4*-specific primers u542 and u544. 10 μl of each sample was electrophoresed through a 1.6% agarose gel and Southern blotted onto nitrocellulose. An *unc-4*-specific oligonucleotide, u4p1, and an *unc-4*-specific probe, u4p, were radiolabeled in the presence of [γ-32P]ATP and polynucleotide kinase and hybridized with the blot at 45°C overnight (Sambrook et al., 1989). The blot was washed at a final stringency of 1× SSC, 0.5% SDS at 45°C for 15 minutes and exposed to X-ray film with an intensifying screen. The linearity of the PCR reaction was established by autoradiography of samples amplified for a range of cycles (Chelly et al., 1990).

**Oligonucleotide primers used for PCR analysis**

u4p1 and u4p4 contain 5′ terminal adaptor EcoRI sites (underlined) that are not present in the *unc-4* genomic sequence. u4p10 includes an adaptor with a BamHI site (underlined).

**RESULTS**

**A 5.5 kb *PstI*-KpnI genomic fragment spans the *unc-4* gene**

We used a microinjection/transformation assay (Mello et al., 1992) to identify genomic DNA that complements the Unc-4 mutant phenotype. Transformation with the cosmid CO7E2, which had been previously shown to include *unc-4* coding sequence (Miller et al., 1992) is sufficient to rescue the Unc-4 mutant phenotype. CO7E2 digested with BamHI, PstI, or ClaI also rescues the Unc-4 mutant phenotype indicating that these restriction sites are not located in the *unc-4* gene. To confirm these results, the 7 kb *PstI*-ClaI fragment was subcloned and shown by microinjection to contain *unc-4* gene activity. Subsequently, we found that the 5.5 kb *PstI*-KpnI fragment (*pNC4-21*) is sufficient to rescue the phenotype whereas a subclone deleted for the region upstream of the *Spel* site does not carry *unc-4* activity (Fig. 2).

**unc-4(+)/lacZ expression in VA motor neurons restores normal movement to *unc-4* mutants**

The *unc-4(+)/lacZ* expression plasmid, *pNC4-23Lz*, was constructed from the presumptive *unc-4* gene regulatory region fused to a cDNA of the *unc-4* coding sequence (Fig. 2). *pNC4-23Lz* was microinjected into the null mutant, *unc-4(wd1)* (Miller et al., 1992); wild-type movement is restored to transformed progeny which indicates that the *unc-4* homeoprotein does function in vivo as a fusion protein with β-galactosidase and that *unc-4(+)/lacZ* expression is likely to have occurred in the appropriate cells.

We have relied upon the instability of the *unc-4(+)/lacZ* transgene to show that Unc-4 rescue is correlated with *unc-4(+)/lacZ* expression in VA motor neurons (Fig. 3). In most cases, transgenic DNA is transmitted as an extrachromosomal.
array in *C. elegans* and is frequently lost from somatic cells during development (Mello et al., 1992). *lacZ*-expressing transgenes may also be inactivated by an unspecified mechanism that is not correlated with cell lineage (data not shown). As a result, a population of mutant animals transformed with a wild-type gene typically displays a range of phenotypes. In this instance, for example, between 18% and 66% of progeny from an *unc-4*(+)*lacZ* transformed *unc-4*(wd1) parent exhibit wild-type or nearly wild-type movement (data not shown).

Individual wild-type or Unc-4 larval animals from the *unc-4*(+)*lacZ* transformed line were picked into separate pools and stained for β-galactosidase activity (Fire et al., 1990). In animals that displayed wild-type movement, most (ave = 7.4) of the VA motor neurons in the ventral nerve cord are *lacZ* positive (Fig. 3A) whereas a significantly smaller number of VA motor neurons (ave = 0.95) are stained in animals exhibiting the Unc-4 movement defect (Fig. 4). The interneurons that provide input to the VA motor neurons do not express the *unc-4*(+)*lacZ* transgene in either wild-type or Unc-4 animals (Fig. 3B). These findings indicate that *unc-4* rescue depends on *unc-4*(+)*lacZ* expression in the VA motor neurons but not in presynaptic interneurons.

It is also significant that the *unc-4*(+)*lacZ* transgene is expressed during the period in which VA motor neurons accept input from interneurons. VA and VB motor neurons are generated in the first larval stage (L1) (Sulston and Horvitz, 1977) and are wired into the ventral nerve cord circuit by the beginning of the L2 (J. White personal communication). We

---

**Fig. 3.** *unc-4*(+)*lacZ* is expressed in VA motor neurons. Anterior is to the left. (A) Ventral view of *unc-4*(wd1) late L1 larva transformed with pNC4-23Lz. X-gal staining is visible in most (nine of ten) VA motor neurons in the ventral nerve cord, and in certain neurons in the preanal ganglion (PAG), retrovesicular ganglion (RVG), and pharynx (see Figs 6, 8). (B) Interneurons that are presynaptic to the VA motor neurons in the wild type (AVA, AVD, AVE) or in an *unc-4* mutant (AVB) are located on the dorsal side (arrow) of the pharynx and are *not* *lacZ* positive. (C) *unc-4*(+)*lacZ* is not expressed in VB motor neurons. Lateral view of posterior ventral nerve cord of L2 larva. Pale blue nuclei are stained with DAPI. VA and DA motor neurons are stained (see Figs 6, 8). Scale bar, 20 μm.

---

**Fig. 4.** Expression of *unc-4*(+)*lacZ* in VA motor neurons restores normal movement to *unc-4* mutant animals. Individual larvae displaying either wild-type or Unc-4 movement were picked from the progeny of an *unc-4*(wd1) animal transformed with the pNC4-23Lz and stained with X-gal. The number of *lacZ*-positive VA motor neurons was determined in randomly selected L2 larvae (n=20) in each category (i.e., wild-type or Unc-4). Only the results for the nine VA motor neurons (VA2-VA10) that are miswired in *unc-4* mutants are shown (White et al., 1992).
have not detected unc-4(+)lacZ expression in VB motor neurons (Fig. 3C). The VA motor neurons, however, are lacZ positive soon after their birth in late L1 larvae and staining generally persists through the L2 stage. The developmentally regulated expression of unc-4(+)lacZ in the VA motor neurons coincides with the temperature sensitive period of an unc-4 temperature sensitive mutant (Miller et al., 1992). Thus, the unc-4(+)lacZ transgene is expressed in VAs during the developmental period in which unc-4(+) function is required to specify proper input to the VA motor neurons.

unc-4(+)lacZ is also expressed in the DA motor neurons (Fig. 3C) although DA neurons are not miswired in unc-4 mutants and appear to function normally (White et al., 1992). In addition, our results show that rescue of the Unc-4 movement defect does not depend on unc-4(+)lacZ expression in the DAs; two of the 20 rescued unc-4(wd1) animals that were analyzed for Fig. 4 do not show lacZ-positive DA motor neurons in the ventral nerve cord (Fig. 3A) and in an additional eight Unc-4-rescued animals only one DA motor neuron was stained (data not shown).

Certain other neurons (SAB, AVF) in the retrovesicular ganglion (RVG) at the anterior end of the ventral nerve cord also stain for lacZ activity (see below). Although, we could not formally rule out the possibility that unc-4 expression in these neurons accounts for rescue of the Unc-4 phenotype, this explanation seems unlikely since these neurons are either generated in embryos in which unc-4 function is not required for normal movement (SAB) (Miller et al., 1992) or because these lacZ-positive cells (AVF) are not known to be components of the motor neuron circuit and do not make synaptic contacts with the VAs (White et al., 1986).

Our approach does not directly demonstrate that the endogenous unc-4 gene is expressed in the VA motor neurons. We have shown, however, that expression of a functional unc-4(+) transgene in the VA motor neurons complements the Unc-4 mutant phenotype. It therefore seems likely that expression of the native UNC-4 protein in the VA motor neurons functions in a similar manner.

**Axonal outgrowth from VA motor neurons is not altered in unc-4 mutants**

Our findings indicate that the absence of unc-4(+) activity in the VA motor neurons accounts for the respecification of VA presynaptic input to that of the VB motor neurons in unc-4 mutants. Because null mutations in other neurogenic homeodomain genes typically result in striking changes in neuronal morphology and axonal outgrowth (Doe et al., 1988; Schmucker et al., 1992; Merrit et al., 1993), we used a chimeric unc-4-lacZ reporter gene (pNC4-76Lz) to visualize the VA motor axons in an unc-4 mutant; the absence of a nuclear localization signal (NLS) in pNC4-76Lz allows the β-galactosidase fusion protein to diffuse into axonal processes where it can be detected by staining with anti-β-galactosidase (Fig. 2).

In the wild-type, VA motor neurons send out anteriorly directed axons whereas VB axons project posteriorly in the ventral nerve cord (White et al., 1986). In unc-4(wd1) larvae expressing the pNC4-76Lz transgene and stained with anti-β-galactosidase, short, anteriorly directed axons can be seen emanating from VA neurons in the late L1 stage (Fig. 5A). These VA axons continue to elongate anteriorly during larval development (Fig. 5B,C) and are indistinguishable from wild-type VA axons (Fig. 8A). In addition to allowing us to capture
glimpses of the VA axons during outgrowth in the ventral nerve cord, the pNC4-76Lz transgene also was useful for establishing that all of the VA motor neurons appear normal in the unc-4 null mutant. The Unc-4 wiring defect was originally determined from reconstructions of separate anterior (VA1-VA3) and posterior (VA10-VA12) regions of the unc-4(e120) ventral nerve cord. Motor neurons VA4-VA9 were not directly examined although the abnormal dorsal coiling of the midbody region in unc-4 mutants indicates that VA4-VA9 are likely to display the unc-4 wiring defect. (White et al., 1992). Now, we have formally shown that VA4-VA9 also appear morphologically normal in an unc-4 null mutant at the level of resolution of the light microscope. Thus, we have firmly established that the polarity of VA axonal outgrowth is not regulated by unc-4.

The endogenous unc-4 transcript is most abundant during embryonic and early larval development

To facilitate the identification of lacZ-positive cells in the embryo, we created a stable unc-4-lacZ strain, wdIs1. Thirteen X-gal-stained neurons were identified in late stage embryos and L1 larvae (Fig. 6); lacZ is expressed in nine DA motor neurons, three SAB motor neurons, and a single pharyngeal neuron, I5. Staining is most intense during late embryogenesis and persists into the first larval stage (Fig. 6D). The intensity of X-gal staining in these embryonically derived motor neurons appears to decrease in the late L1 but can be detected in older larvae (Fig. 3C) and in adults (Fig. 9) in some cases.

In order to determine if the endogenous unc-4 gene is also transcribed during this period, we used a reverse transcription-PCR assay to measure unc-4 mRNA in wild-type (N2) animals. As shown in Fig. 7, the endogenous unc-4 transcript is most abundant in embryos and young larvae and begins to diminish in the L2 stage. The relative abundance of the unc-4 transcript in C. elegans development parallels the time course of unc-4-lacZ expression in embryonic and larval motor neurons and therefore supports the hypothesis that the endogenous unc-4 protein also functions in these neurons during these developmental periods.
unc-4-lacZ is expressed in all A-type motor neurons

*C. elegans* motor neurons have been grouped into classes on the basis of morphology and connectivity. The A-type class includes 24 motor neurons; nine DA and three SAB motor neurons are generated in the embryo and 12 VAs arise after hatching in the first larval stage. The DA, SAB, and VA motor neurons exhibit anteriorly directed axonal processes and accept synaptic input from a common set of interneurons (AVA, AVD, AVE) (White et al., 1986). *unc-4-lacZ* is expressed in all of these A-type motor neurons (Figs 3, 5, 6).

VA motor neurons send out anteriorly directed axonal processes in the ventral nerve cord and short posterior dendrites. DA axons grow out along predictable paths (commissures) to the dorsal side of the animal then project anteriorly. The SAB motor neurons are located in the RVG just ventral to the posterior bulb of the pharynx and send out anteriorly directed processes that enter the ventral sublateral cords (SABVR, SABVL) and the dorsal sublateral cords (SABD) (White et al., 1986). The axonal trajectory and morphology of each of these classes can be visualized in the light microscope in *pNC4-76Lz* transgenic animals (Fig. 8).

The VAs and DAs are probably excitatory motor neurons and perform similar but complementary functions (White and Chalfie, 1988). VA motor neurons innervate ventral muscles and DA motor neurons provide output to dorsal muscles (White et al., 1986); both are required for backward movement (Chalfie et al., 1985). The DA motor neurons are not miswired in *unc-4* mutants, however, and appear to function normally, as *unc-4* animals are able to coil dorsally (DA function) but not ventrally (VA function) when touched on the head (White et al., 1992). Because the SABs have not been fully reconstructed in an *unc-4* mutant (White et al., 1992) it is not known if presynaptic input is affected by the absence of *unc-4* activity.

We did not detect any significant changes in SAB morphology in a *unc-4(wd1)* background (data not shown). The significance of *unc-4-lacZ* expression in the pharyngeal neuron, I5, is unknown although pharyngeal pumping is not perturbed in *unc-4(e120)* (Leon Avery, personal communication).
unc-4-lacZ expression in postembryonic neurons is correlated with cell lineage

We have also detected unc-4-lacZ expression in a pair of postembryonically derived neurons, AVFR and AVFL, that are not motor neurons but which arise from a pattern of cell divisions that is homologous to the VA motor neuron lineage. Thirteen precursor cells or P-cells (P0 - P12) give rise to five classes of motor neurons during the first larval stage (Sulston and Horvitz, 1977). Eleven VA motor neurons (VA2 - VA12) are born as the most anterior daughters (Pn.aaaa) of P2 - P12. The most anteriorly generated daughters of P0 and P1, however, are the AVF interneurons AVFR and AVFL. (VA1 arises from P0 as the P0.pa daughter and P1 does not produce a VA motor neuron.) The VA motor neurons and the AVF interneurons are lacZ positive (Fig. 9). Thus, unc-4-lacZ is expressed in the most anterior daughters of all postembryonic P-cells and in all of the VA motor neurons.

The function of the AVF interneurons is not known (White et al., 1986). The AVFs make a limited number of synaptic contacts and these are apparently normal in the ventral nerve cord and RVG in unc-4(e120) (J. White, personal communication). No significant changes in axonal AVF morphology were observed by unc-4-unc-76-lacZ staining of unc-4(wd1) (data not shown).

DISCUSSION

The C. elegans unc-4 gene encodes a homeodomain protein (Miller et al., 1992) that specifies the pattern of synaptic input to VA motor neurons in the ventral nerve cord (White et al., 1992). In unc-4 mutants, VA motor neurons assume the pattern of synaptic input normally reserved for their VB sister cells; the loss of normal input to the VA motor neurons blocks reverse locomotion. Our results described here show that normal movement is restored to an unc-4 mutant in which the unc-4(+)/lacZ transgene is expressed in the VA motor neurons. Because the interneurons that provide input to the VA and VB motor neurons are not lacZ positive, we conclude that unc-4(+)/lacZ expression in the VA motor neurons is sufficient to rescue the Unc-4 mutant phenotype.

In animals expressing the wild-type unc-4(+) gene, reverse locomotion requires input to the VA motor neurons from interneurons AVA, AVD and AVE (Chalfie et al., 1985). It is therefore reasonable to conclude that these connections are restored in transformed unc-4 mutant animals expressing the unc-4(+)lacZ transgene in the VA motor neurons. It seems equally reasonable to conclude that the endogenous unc-4 gene is also expressed in the VA motor neurons to specify proper presynaptic input in the wild-type animal (Fig. 10). It is formally possible that undetectable levels of unc-4(+)/lacZ activity in other cells could be sufficient to rescue the Unc-4 movement phenotype but this explanation seems less plausible than the model we have proposed.

Our results show that unc-4 is expressed in both embryos and larvae but that synaptic input to the VA motor neurons is strictly dependent on postembryonic unc-4 activity (Fig. 11). Temperature shift experiments with an unc-4 temperature sensitive mutant have defined an unc-4 temperature sensitive period (TSP) that overlaps the interval in which VA motor neurons accept input from interneurons (Miller et al., 1992). This finding may be interpreted to mean that the wild-type pattern of input to the VA motor neurons depends on unc-4 function during larval development but does not require unc-4 activity in the embryo in which unc-4-lacZ expression is detected in DA, SAB, and I5 neurons. We do observe X-gal staining in older larvae and occasionally in adults (Fig. 9A) but because the unc-4 TSP does not extend beyond the L3 stage we attribute this residual activity to the perdurance of the unc-4-lacZ fusion protein. This interpretation is also consistent with
steady state levels of unc-4 mRNA which are highest in embryos and early larvae but diminish to background levels after the L2 stage. The unc-4 TSP does indicate, however, that the adult pattern of inputs to the VA motor neurons depends on unc-4 function for a finite period after these connections are initially established at the end of the L1 stage (Miller et al., 1992). This observation may be indicative of a period of neuronal plasticity in which an initial pattern of inputs to the VA motor neurons can be replaced with an alternative set of synapses if unc-4 activity is not maintained.

Our findings predict that the unc-4 homeoprotein controls a specific feature of the VA motor neurons that distinguishes them from their VB sisters thus allowing recognition by appropriate interneurons for synapse formation (Fig. 10). Given that unc-4 is not required for the expression of other VA traits such as axonal morphology and polarity (White et al., 1992), it seems likely that unc-4 controls a limited number of targets and that these downstream genes could be directly involved in synaptic choice (Miller et al., 1992). Dominant mutations in the unc-37 locus are allele-specific suppressors of a point mutation in the unc-4 homeodomain. In addition, the loss-of-function mutation, unc-37(e262), produces a movement defect that is strikingly similar to that of unc-4 null mutants. On the basis of these genetic data, we have proposed that unc-37 either corresponds to an unc-4 target gene or encodes a cofactor protein that physically interacts with the unc-4 homeodomain (Miller et al., 1993). In either case, unc-37 would have a key role in the unc-4-dependent specification of synaptic input to the VA motor neurons.

Mutations in other genes that encode transcription factors have been shown to affect neural connectivity. In all of these cases, however, neuronal morphology as well as synaptic specificity are affected (Doe et al., 1988; Schmucker et al., 1992; Merrit et al., 1993). Ectopic expression of the Drosophila gene, pox neuro, for example, is accompanied by morphological and functional transformation of nascient mechanosensory neurons into chemosensory neurons. Thus, pox neuro is likely to regulate a suite of downstream genes which act together to specify the differentiation of a chemosensory neuron (Nottebohm et al., 1992). In contrast, unc-4 appears to control a subset of traits expressed by the VA motor neurons and is therefore predicted to regulate a much smaller group of target loci that govern neural specificity but not axonal morphogenesis (Miller et al., 1992).

The VA, DA, and SAB motor neurons constitute the A-class of motor neurons (White et al., 1986). Each of the A-type motor neurons receive synaptic inputs from a common set of interneurons (AVA, AVD, AVE) and are morphologically similar with neuronal morphology as well as synaptic input to the DA motor neurons is not altered in an unc-4 mutant (White et al., 1992) although unc-4-lacZ is expressed in these cells. Similarly, three of the twelve VA motor neurons in the ventral nerve cord (VA1, VA11, VA12) are not miswired by the unc-4 mutation whereas the pattern of synaptic input to nine of the twelve VA motor neurons (VA2-VA10) is homeotically transformed to that of the VB motor neurons. Thus, synaptic input to a subgroup of A-type motor neurons may be established by an alternative genetic pathway that does not depend on unc-4 activity (White et al., 1992). It may be significant that all of the VA motor neurons for which unc-4 does specify synaptic input are born with VB sister cells in the motor neuron cell lineage, whereas the A-type motor neurons in which unc-4 does not determine input do not have B-type motor neuron sisters (Fig. 9C) (Sulston and Horvitz, 1977). Perhaps unc-4 function is specifically required to supersede a B-type motor neuron default program triggered in VA daughters sharing a common precursor with a VB neuroblast.
The unc-4-lacZ reporter genes are selectively expressed in 27 neurons. With the exception of the pharyngeal neuron, 15, all of the unc-4-lacZ-positive neurons are either functionally and morphologically similar (the A-type motor neurons DA, SAB, VA) or arise from a similar lineage (AVF, VA). The unc-4 upstream flanking region appears to respond to an A-type (1976). The pharynx of

REFERENCES


(accepted 20 May 1995)