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

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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* homeoprotein is fused to the reporter enzyme, β-galactosidase (β-gal), and 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

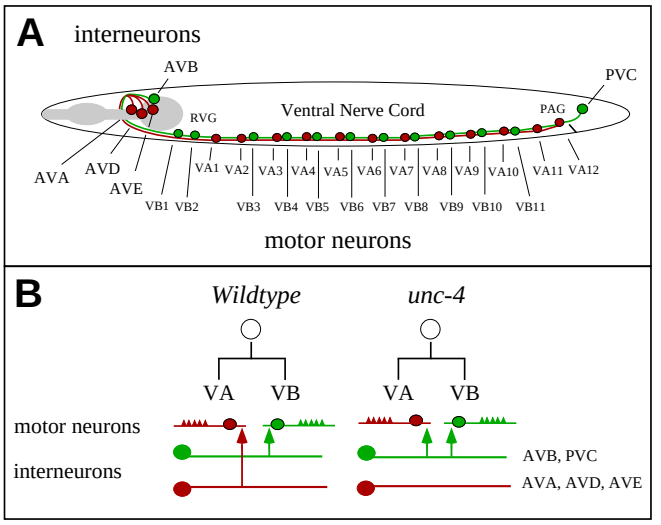

Fig. 1. The *unc-4* gene defines the pattern of synaptic input to VA motor neurons. (A) Axons from interneurons in the head (AVA, AVD, AVE, AVB) and in the tail (PVC) project into the ventral nerve cord during embryonic development. Twelve VA motor neurons and eleven VB motor neurons are innervated by separate sets of interneurons (indicated by color coding). (B) Most of the VA and VB motor neurons are derived from a common precursor in the postembryonic motor neuron lineage. VA and VB axons adopt opposite trajectories with VAs projecting anteriorly and VBs posteriorly. In the wild type, VA motor neurons accept input from interneurons AVA (gap junction and chemical synapse), AVD, AVE (chemical synapse) whereas the VBs are connected to AVB (gap junction) and PVC (chemical synapse). In null mutant *unc-4(e120)*, VA motor neurons retain their normal morphology but assume the pattern of synaptic input normally reserved for their VB sisters (gap junction from AVB). These connections (arrows) are made *en passant* between parallel-oriented processes and do not require axonal branching (White et al., 1992).

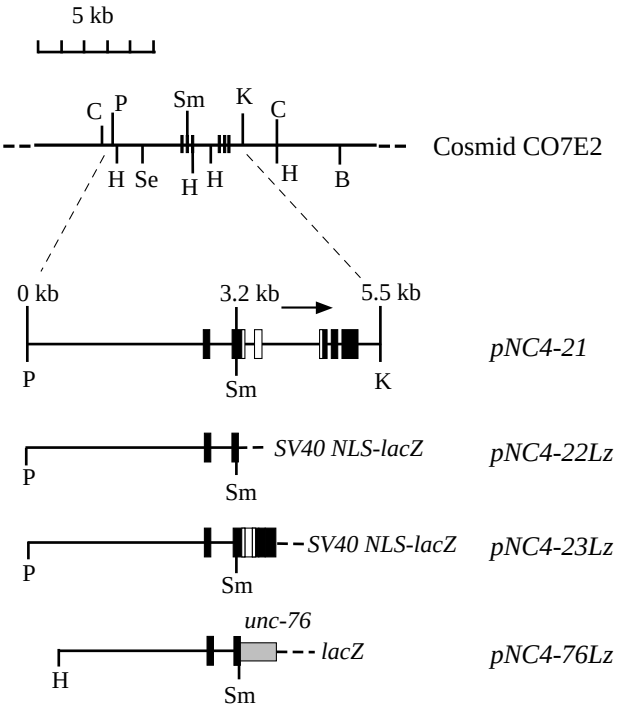

Fig. 2. *unc-4* genomic region and plasmids used to define *unc-4* transcription unit and pattern of expression. *unc-4* coding sequence is shown as black rectangles. White rectangles depict the *unc-4* homeodomain. Arrow refers to direction of transcription. SV40 NLS refers to a nuclear localization signal. The *unc-76* protein domain in *pNC4-76Lz* excludes X-gal staining from the nucleus. Restriction sites are: B, *Bam*H1; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Se, *Spe*I; Sm, *Sma*I.

Center which is funded by the NIH Center for Research Resources: *unc-37(e262) I, rol-6(e187) II, dpy-20(e1282) IV*.

Molecular biology and construction of *lacZ* plasmids

The 5.5 kb *Pst*I-*Kpn*I 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 *Pst*I-*Sma*I fragment from *pNC4-21* was subcloned into *Pst*I-*Sma*I-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 B*am*HI site (Miller et al., unpublished data). A 580 bp *Sma*I-*Bam*HI cDNA fragment derived from the u4p9/u4p10 PCR product was ligated with the 3.2 kb *Pst*I-*Sma*I genomic fragment from the *unc-4* upstream region and *Pst*I-*Bam*HI 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 *Hin*dIII-*Sma*I fragment from the *unc-4* upstream region (Fig. 2) was ligated into *Hin*dIII-*Sma*I cut plasmid *p76-L18*, which contains a 0.6 kb *unc-76* cDNA 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. F_2 progeny were evaluated for cotransformation; tester DNA was scored as positive if a majority of the plates giving rise to non-Dpy F_2 progeny were also non-Unc.

unc-4-lacZ transgenic lines were obtained by picking non-Dpy F_2 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 (*wdIs1*) 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 (diamidinophenolindole) 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 *wdIs1* 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); I5 (fig. 23e 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 innoculated 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 floated on 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-54*-specific primers, u542 and u544, in a total volume of 50 μl. *unc-54* mRNA was simultaneously amplified in each reaction as an internal control. *unc-54* mRNA is 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-54*-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-54*-specific probe, u542, were radiolabeled in the presence of [γ-^{32}P]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 *Eco*RI sites (underlined) that are not present in the *unc-4* genomic sequence. u4p10 includes an adaptor with a *Bam*HI site (underlined).

u4p1: 5′-GGAATTCTCTCACTCGAACCATTTCGG-3′
u4p2: 5′-ATTCCCACCAGGCTGAGTGAT-3′
u4p4: 5′-GGAATTCTGGCAACTGGAAGAGCTCGA-3′′
u4p9: 5′-GATCGGTGCACTGCATGC-3′
u4p10: 5′-GTCGACGGATCCTCAGCAACCGTAGTCAATGC-3′

The positions of the *unc-54*-specific primers in the genomic *unc-54* gene sequence are based upon the numbering system of Karn et al. (1983). The primer u542 spans an intron as indicated.

u542: 7356-7370, 7428-7433 5′-ATCCAAGCCATTCATGCCGAC-3′
u544: 8108-8128 5′-ATGCGGAGCTCTGGAGTCCTG-3′

RESULTS

A 5.5 kb *Pst*I-*Kpn*I 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. C07E2 digested with *Bam*HI, *Pst*I, or *Cla*I 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 *Pst*I-*Cla*I fragment was subcloned and shown by microinjection to contain *unc-4* gene activity. Subsequently, we found that the 5.5 kb *Pst*I-*Kpn*I fragment (*pNC4-21*) is sufficient to rescue the phenotype whereas a subclone deleted for the region upstream of the *Spe*I 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

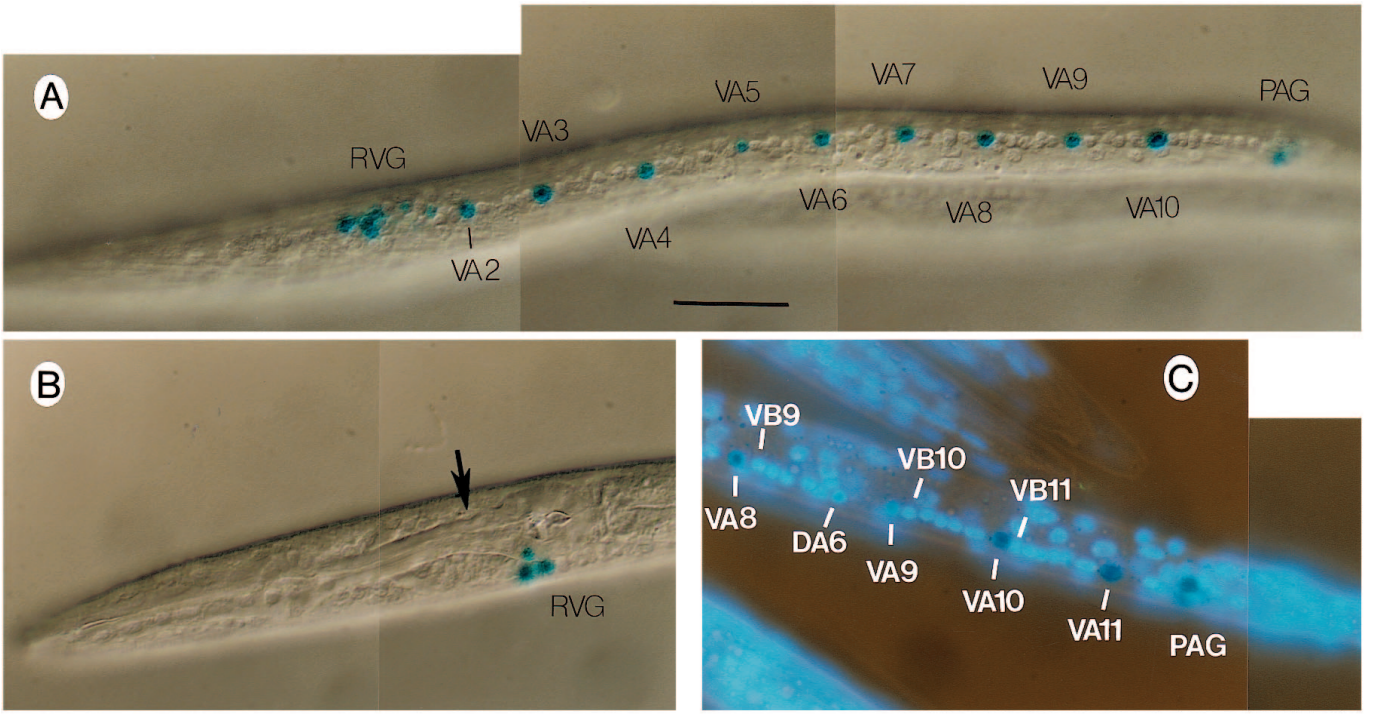

Fig. 3. *unc-4*(+)*lacZ* is expressed in VA motor neurons. Anterior is to the left. (A) Ventral view of *unc-4(wd1)* late L1 larva transformed with *pNC4-23Lz*. X-gal staining is visible in most (nine of ten) VA motor neurons in the ventral nerve cord, and in certain neurons in the preanal ganglion (PAG), 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.

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

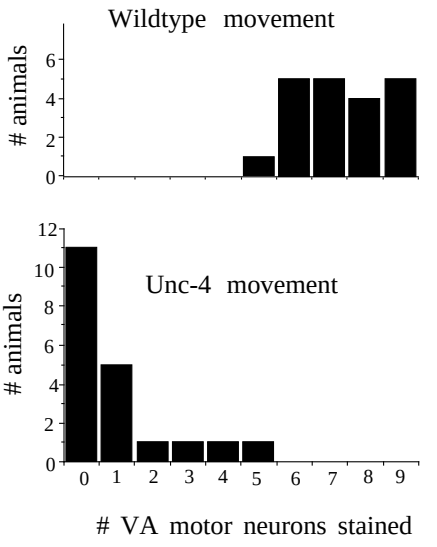

Fig. 4. Expression of *unc-4*(+) *lacZ* in VA motor neurons restores normal movement to *unc-4* mutant animals. Individual larvae displaying either wild-type or Unc-4 movement were picked from the progeny of an *unc-4(wd1)* animal transformed with the *pNC4-23Lz* and stained with X-gal. The number of *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

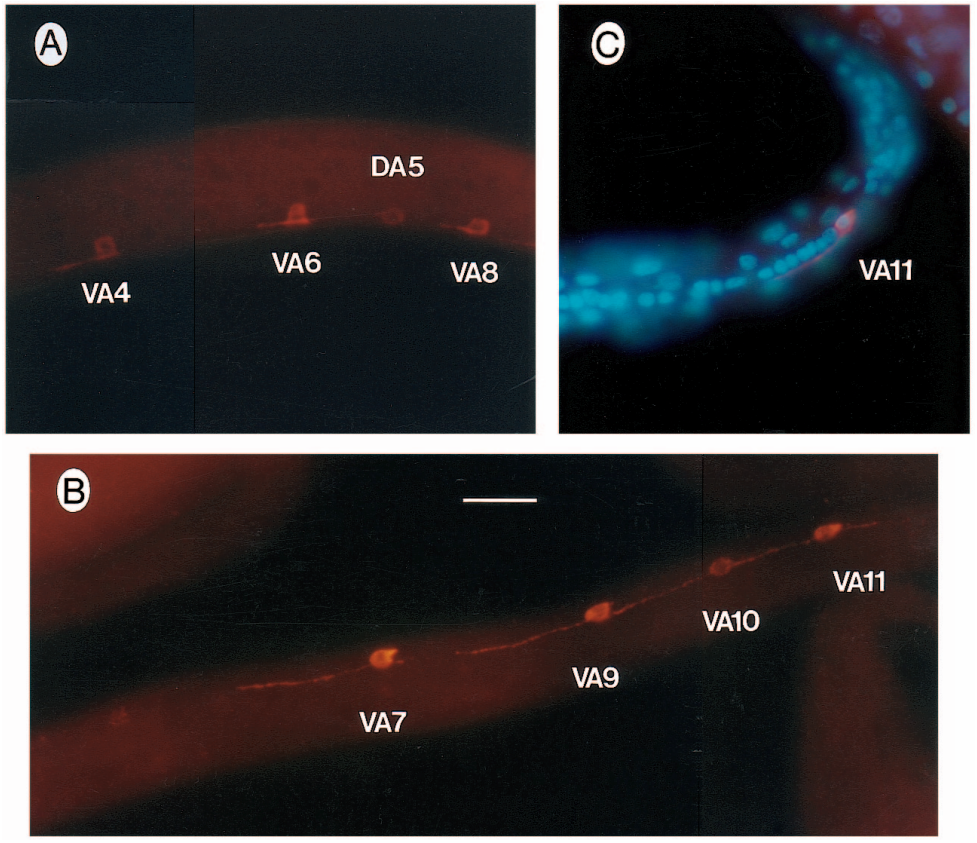

Fig. 5. VA axonal outgrowth is not perturbed by the *unc-4* mutation. *unc-4(wd1)* animals expressing the *pNC4-76Lz* transgene were stained with anti-β-galactosidase. Anterior is to the left. (A) Lateral view of late L1 larva shortly after the emergence of anteriorly directed axons from VA motor neurons. (B) Ventrolateral view of early L2 larva. (C) Lateral view of late L1. DAPI stains (light blue) cell bodies of other motor neurons in the ventral nerve cord (5). Scale bar, 10 μm.

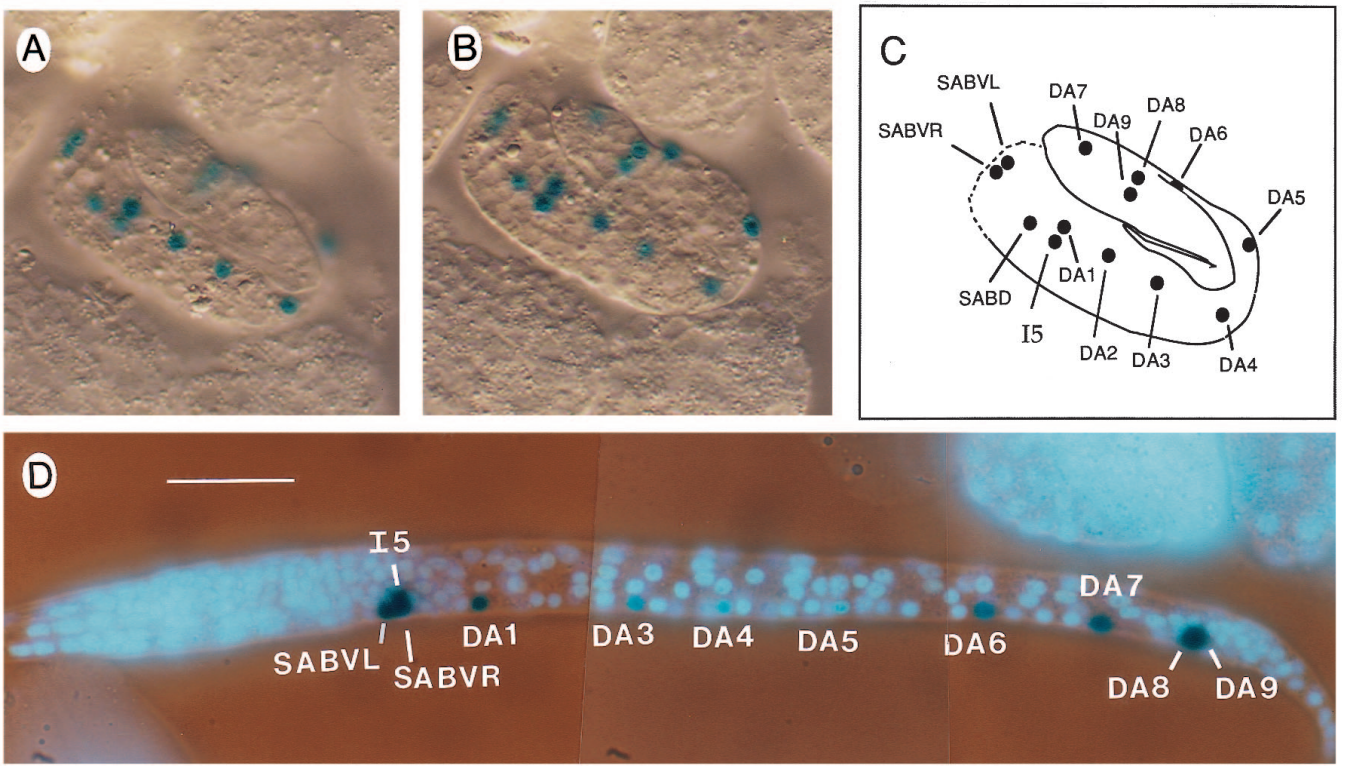

Fig. 6. *unc-4-lacZ* is expressed in embryonically derived motor neurons. Anterior is to the left. (A,B) Two focal planes showing X-gal-stained neurons in a four-fold embryo from *pNC4-22Lz* integrated line, *wdIs1*. (C) Summary of X-gal staining pattern in embryo shown in A and B. The anterior end of the embryo is folded back under the body at the dashed line and is not shown. Thirteen neurons are stained: SABVR, SABVL, SABD in the RVG; DA1-DA9 in the RVG, ventral nerve cord, and PAG, I5 in the pharynx. (D) Pattern of*unc-4-lacZ* expression in newly hatched L1 larva. In this case, two neurons, SABD and DA2, do not stain. Light blue nuclei are counterstained with DAPI. Scale bar, 20μm.

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.

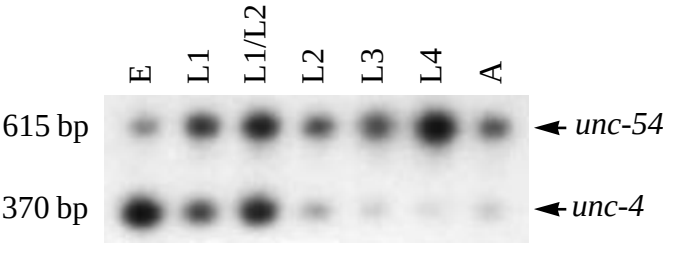

Fig. 7. Developmental profile of *unc-4* transcription. Total RNA was isolated from embryos (E), from synchronized populations of developing larvae (L1- L4), and from young adults (A). The L1/L2 timepoint was obtained from a mixed population of late L1 and early L2 larvae. Reverse transcription-PCR was employed to detect the *unc-4* and *unc-54* transcripts.

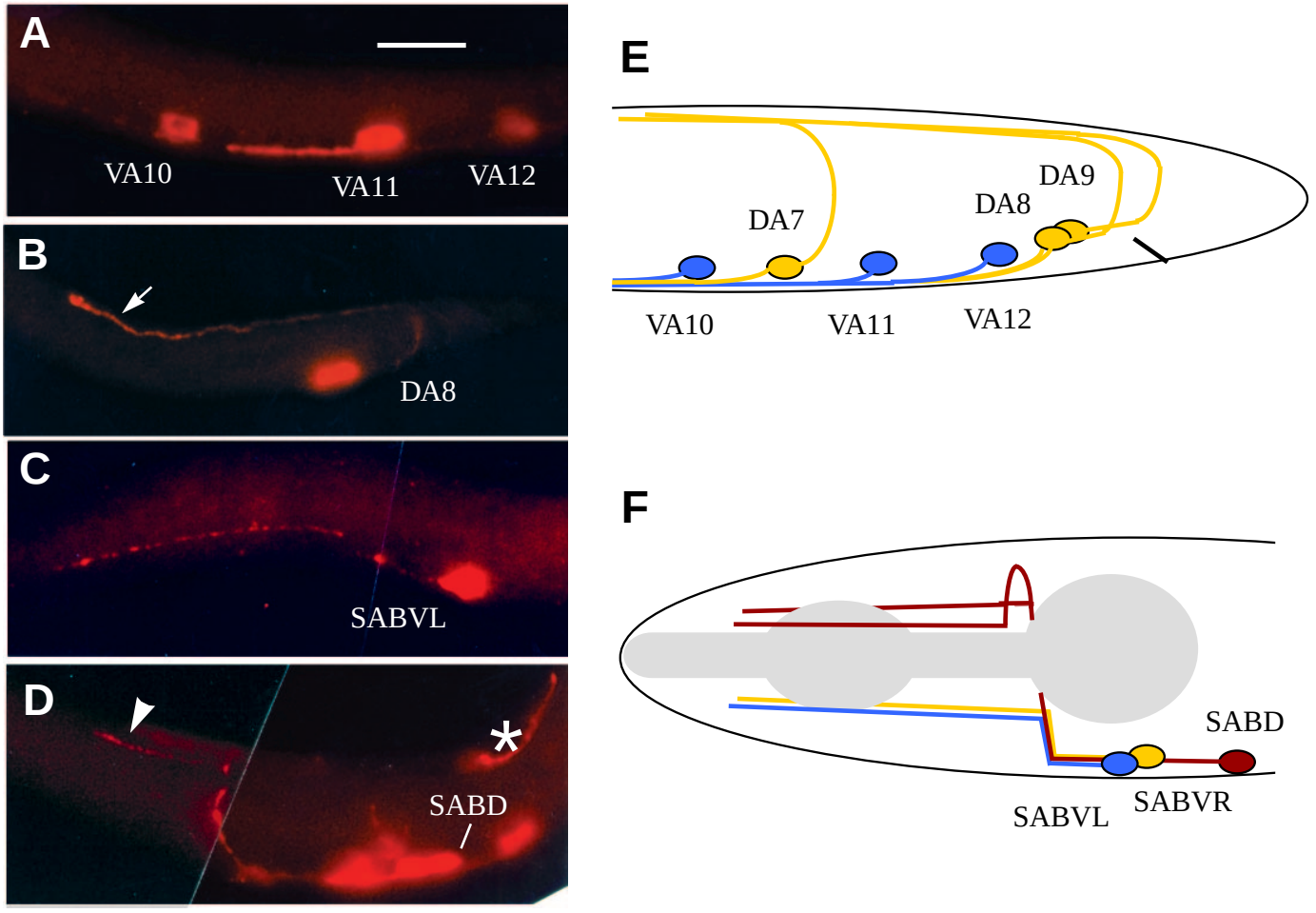

Fig. 8. *unc-4-lacZ* is expressed in A-type motor neurons. Expression of *pNC4-76Lz* transgene was detected by staining with anti-β-galactosidase. Anterior is to the left. (A) Tail region of late L1 larva (left view) showing anteriorly directed VA11 axon. (B) Tail region of early L1 (left view) showing DA8 commissure and anteriorly directed axon in dorsal nerve cord (arrowhead). (C) Head region of early L1 (left view) with anteriorly directed axon from SABVL in left subventral cord. (D) Head region of early L1 (right view) showing SABD commissure and anteriorly directed axon in right subdorsal cord (arrowhead). Asterisk denotes DA2 axon in dorsal cord. For C and D, photos of two separate focal planes were spliced together to show axonal morphology in a single image. Scale bar, 10 μm. (E) Posterior end of ventral nerve cord (left view). (F) Head region (left side).

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).

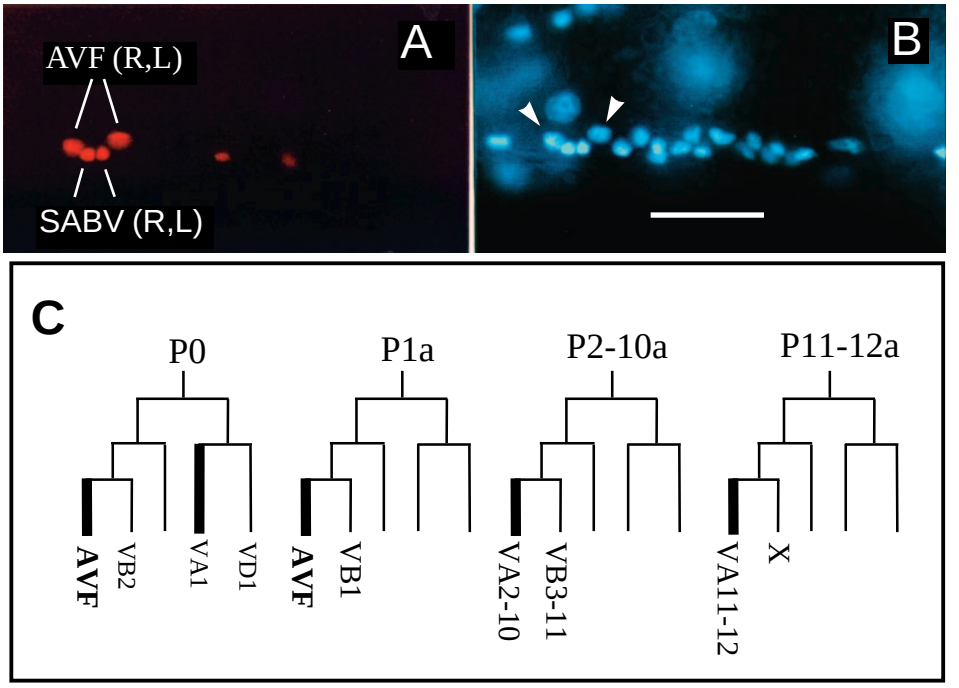

Fig. 9. *unc-4-lacZ* is expressed in all VA motor neurons and in all of the most anterior daughters of the P-cells. Anterior is to the left. (A) Adult stained with anti-β-galactosidase to reveal *lacZ*-positive neurons in the RVG. One (probably VA1) of the seven *unc-4-lacZ* expressing neurons in the RVG is not stained in this image. (B) DAPI-stained image of A to show all neurons in the RVG. Arrowheads denote AVF(R,L) cell nuclei. Scale bar, 20 μm. (C) Cell lineage of *unc-4-lacZ*-expressing neurons. Each horizontal line represents a cell division and each vertical line represents a single cell. The thickened lines identify *lacZ*-positive neurons. Thirteen precursor cells or P-cells (P0-P12) give rise to twelve VA motor neurons and to two AVF neurons in the L1 stage.

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

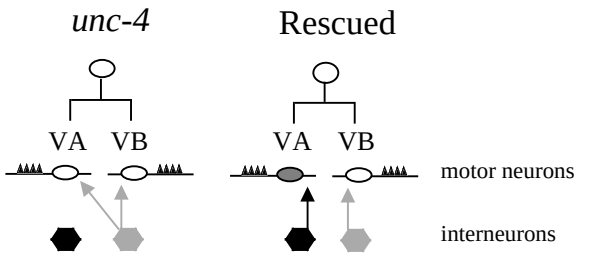

Fig. 10. A model of *unc-4* action: *unc-4* expression in VA motor neurons specifies presynaptic input. VA and VB motor neurons arise as sister cells from a common precursor but extend axons in opposite directions in the ventral nerve cord (VA, anterior and VB, posterior). In *unc-4* mutants, VA motor neurons exhibit normal morphology but accept the VB pattern of synaptic input. *unc-4* mutant animals expressing the *unc-4-lacZ* homeoprotein in a majority of VA motor neurons show improved or normal movement which indicates that the wild-type pattern of synaptic input to the VAs has been restored. Black hexagon represents interneurons AVA, AVD, AVE and gray hexagon, interneuron AVB.

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 intial 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 nascent 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 anteriorly directed axons. The wild-type pattern of 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.

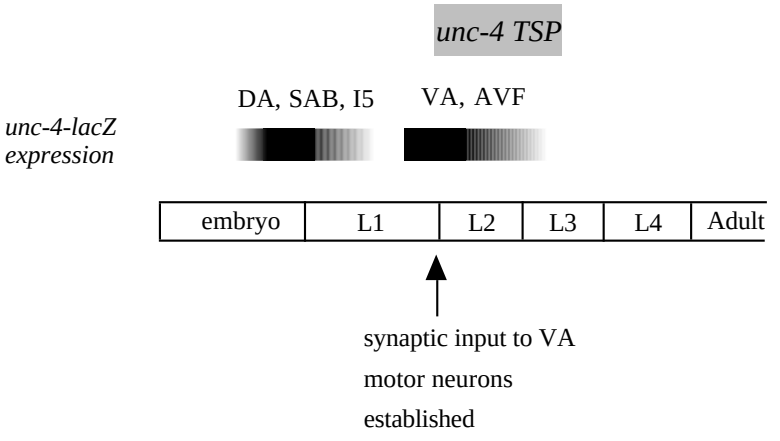

Fig. 11. Temporal pattern of *unc-4-lacZ* expression in ventral cord neurons. *LacZ* expression is first detected in DA, SAB, and I5 neurons immediately after morphogenesis begins at the approximate midpoint of embryonic development and diminishes during the L1. β-galactosidase activity is detected in the VA motor neurons after their birth in the late L1 stage. *unc-4-lacZ* expression in the VA motor neurons overlaps the *unc-4* temperature sensitive period (TSP) in which *unc-4* function is required to establish proper synaptic input to VA motor neurons in the temperature sensitive strain *unc-4(e2322ts)*.

The *unc-4-lacZ* reporter genes are selectively expressed in 27 neurons. With the exception of the pharyngeal neuron, I5, 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 motor neuron developmental program and may therefore include binding sites for transcription factors that also control the expression of other motor neuron class-specific traits. The selected expression of *unc-4-lacZ* in all of the most anterior daughters of the postembryonic P-cells is indicative of an additional set of *cis*-acting sequences that respond to cell-lineage specific cues.

Previously, axonal processes from the *C. elegans* excitatory motor neurons could only be observed in the electron microscope (White et al., 1986). We have utilized an *unc-4-lacZ* reporter contruct to confirm that the morphology and polarity of VA motor neurons is not perturbed in *unc-4* mutants. Using this approach, we have also shown that the loss-of-function mutation, *unc-37(e262)*, has no visible effect on VA morphology, which favors the hypothesis that the movement defect in *unc-37(e262)* is also a consequence of improper input to VA motor neurons and is not a result of misplaced or abnormal VA axons (Miller et al., 1993).

In summary, we have shown that expression of a functional *unc-4-lacZ* reporter gene in the *C. elegans* VA motor neurons is sufficient to rescue the movement defect conferred by inappropriate synaptic input to the VA motor neurons in *unc-4* mutants. We have therefore proposed that the endogenous *unc-4* gene is normally expressed in the VA motor neurons to specify proper presynaptic input. Our findings indicate that neural specificity can be controlled at the level of transcription. In contrast to other known neurogenic homeodomain genes which govern multiple neuronal traits, *unc-4* activity appears to account for a subset of VA-specific traits and may therefore control a small number of target genes that are directly involved in synaptic choice. A major objective for the future is to identify the genes that are regulated by the *unc-4* homeodomain.

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