Control of Neural Development and Function in a Thermoregulatory Network by the LIM Homeobox Gene *lin-11*

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We show here that the *lin-11* LIM homeobox gene is expressed in nine classes of head, ventral cord, and tail neurons and functions at a late step in the development of a subset of these neurons. In a *lin-11* null mutant, all *lin-11*-expressing neurons are generated. Several of these neurons, however, exhibit neuroanatomical as well as functional defects. In the lateral head ganglion, *lin-11* functions in a neural network that regulates thermosensory behavior. It is expressed in the AIZ interneuron that processes high temperature input and is required for the function of AIZ in the thermoregulatory neural network. Another LIM homeobox gene, *ttx-3*, functions in the antagonistic ther-

moregulatory interneuron AIY (Hobert et al., 1997). Thus, distinct LIM genes specify the functions of functionally related antagonistic interneurons within a neural network dedicated for thermoregulatory processes. Both *ttx-3* and *lin-11* expression are maintained throughout adulthood, suggesting that these LIM homeobox genes play a role in the functional maintenance of this neural circuit. We propose that particular LIM homeobox genes specify the distinct features of functionally related neurons that generate patterned behaviors.

Key words: LIM homeobox gene; neurogenesis; thermotaxis; neural function; axon pathfinding; axon fasciculation

Genetic analysis of animals showing behavioral or neuroanatomical defects has identified pathways that control the generation and functional specification of neurons (Desai et al., 1988; Ruvkun, 1997). Such studies have revealed sequential steps to neurogenesis that are mediated by conserved regulatory factors. Homeobox genes represent a class of regulatory factors that control various stages of neurogenesis in *Caenorhabditis elegans* and other organisms (Chalfie, 1993; Manak and Scott, 1994). For example, the *unc-86* POU homeobox gene acts early in neural development to couple cell lineage to neuroblast identity (Finney and Ruvkun, 1990). After determination of neuroblast fate in *C. elegans*, certain neuroblast migrations are regulated by a homeobox gene (Salser and Kenyon, 1992). The determination of the pattern of presynaptic input or neurotransmitter identity is also under control of homeobox genes (Miller et al., 1992; White et al., 1992; Jin et al., 1994).

Dedicated neural networks that subserve specific behaviors have been identified in *C. elegans*. The cellular components of the neuronal circuitry evoking the touch and tap responses and the neuronal circuitry that regulates the response to temperature have been characterized in detail, revealing the existence of dedicated sensory neurons, interneurons, and motor neurons

(Chalfie et al., 1985; Mori and Ohshima, 1995; Wicks and Rankin, 1995). A genetic analysis of these behaviors has identified several regulatory factors that are required to specify functionally these neural circuits. For example, the *mec-3* LIM homeobox gene is required for the specification of touch neurons within the touch circuit (Way and Chalfie, 1988), whereas the *ttx-3* LIM homeobox gene acts to control the function of an interneuron in the thermoregulatory circuit (Hobert et al., 1997).

LIM homeobox genes represent a particular subclass of homeobox genes that encode proteins characterized by the presence of a DNA-binding homeodomain and two LIM domains, each of which bears two Zn-finger-like motifs (for review, see Dawid et al., 1995). LIM homeobox genes have been identified across phylogeny and can be grouped into defined subclasses (Fig. 1). The analysis of LIM homeobox genes in *C. elegans* and *Drosophila* has suggested a critical role for this class of genes in neurogenesis. The *Drosophila apterous* and *islet* genes control axon pathfinding of particular neurons in the CNS (Lundgren et al., 1995; Thor and Thomas, 1997). The *C. elegans* LIM homeobox genes *mec-3* and *ttx-3* are each necessary for the differentiation of particular neuronal cell types; the continuous expression of these genes throughout adulthood suggests that they are also required for neural maintenance (Way and Chalfie, 1991; Hobert et al., 1997). Similarly, two other *C. elegans* LIM homeobox genes identified from the genome sequence display a neural restricted expression pattern that is maintained throughout adulthood (O. Hobert and G. Ruvkun, unpublished observations).

The *lin-11* LIM homeobox gene was first identified by the defects in ectodermal cell lineage asymmetry caused by *lin-11* loss-of-function mutations (Ferguson and Horvitz, 1985; Freyd et al., 1990). Given the involvement of other LIM homeobox genes in neurogenesis, we sought to determine whether *lin-11* also plays a role in neural development or neural function. We find that *lin-11* is expressed in a specific set of postmitotic neurons. *lin-11* is not required for the generation of these neurons but has a role

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Figure 1. Subclasses of LIM homeobox genes. The nomenclature used for vertebrate LIM homeobox genes has been proposed by Dawid et al. (1995). Subclasses for which only one member has been identified to date (e.g., *C. elegans* MEC-3) are not shown. The dendrogram was constructed with the pileup algorithm in the Genetics Computer Group software package using only the homeodomain sequences of the LIM homeodomain proteins because the evolutionary constraints on variations within this functional domain are most pronounced and thus produce the most significant relationship among various homeodomain family members (Bürglin, 1995). CeISL-1 represents an ISLET homolog recently identified in *C. elegans* (Hobert and Ruvkun, unpublished observations). BK64 and BK87 represent only partially characterized *Drosophila* LIM homeobox gene sequences retrieved from a DNA-binding screen (Kalionis and O'Farrell, 1993). Because most of the *C. elegans* genome has been sequenced, it is worthwhile to note that no LIM homeobox gene subclass has more than one *C. elegans* member to date. Not all vertebrate orthologs are shown. AP, Apterous; C.e., *C. elegans*; D.m., fruit fly; G.g., chicken; H.r., ascidian; H.s., human; M.m., mouse; and X.l., frog.

in their functional specification. One of these neurons has a function that is related to the function of a neuron specified by the LIM homeobox gene *ttx-3*. The interneurons AIZ and AIY act antagonistically in the neural network that subserves thermoregulatory behavior, and the LIM genes *lin-11* and *ttx-3*, respectively, are necessary for events late in the development of each neuron. These findings indicate that LIM homeobox gene paralogs may serve homologous regulatory functions in the development of related neurons.

MATERIALS AND METHODS

Strains and genetic procedures. The strains used in this study are wild-type *C. elegans* Bristol strain (N2), CB1467 [*him-5(e1467)*], MT633 [*lin-11(n389)*; *him-5(e1467)*], GR1052 [*unc-86(n846)*], and CB1372 [*daf-7(e1372)*]. *lin-11(n389)* represents a null mutation in which the entire coding region of *lin-11* is deleted (Freyd et al., 1990). Double mutant strains were constructed as follows: the *daf-7(e1372)*; *lin-11(n389)* double mutant strain was constructed by mating *lin-11(n389)*; *him-5(e1467)* males with *daf-7(e1372)* hermaphrodites. Nondauer *lin-11*, *daf-7* heterozygous cross-progeny was identified by raising animals at 25°C. F2 animals homozygous for *daf-7* were identified at 25°C and recovered at 15°C, and homozygous *lin-11* adults were scored for the *lin-11*-associated egg-laying defect.

Expression constructs and generation of transgenic animals. The *plin-11-ABCDE-GFP* reporter gene was constructed by subcloning a genomic 10 kb fragment into the pPD95.75 vector, creating a translational fusion of a *Bam*HI site within the fifth exon of *lin-11* just upstream of the homeobox with the green fluorescent protein (GFP) coding region. The *plin-11-CDE-GFP*, *plin-11-DE-GFP*, and *plin-11-E-GFP* constructs were injected as purified restriction fragments obtained from the original

plin11-ABCDE-GFP construct using the *Apa*LI site at nucleotide position –212 of the ATG start codon (for *plin-11-CDE-GFP*), the *Bst*EII site at position –1466 (for *plin-11-DE-GFP*), or the *Bgl*II site at position –2564 (for *plin-11-E-GFP*) plus the *Eag*I site downstream of the GFP 3'-untranslated region. The plasmid or restriction fragment DNA was injected at 50 ng/μl into N2 wild type using pRF-4 (harboring a dominant *rol-6* mutation) as an injection marker (100 ng/μl). Multiple independent transgenic lines were examined for expression. The *plin-11-ABCDE-GFP* and *plin-11-DE-GFP* extrachromosomal arrays were integrated using a Stratalinker 1800 UV light source at 300 J/m² and were back-crossed several times. Neuroanatomical defects (see Fig. 4) were identified by crossing the integrated *plin-11-DE-GFP* reporter array from wild-type into *lin-11(n389)* null mutant animals. Because this allowed the comparison of reporter strains that only differed in *lin-11* gene activity and otherwise were completely isogenic, artifacts induced by the reporter array could be excluded.

Cell identifications and scoring of neuroanatomical defects. Identification of neurons that express *lin-11-GFP* was done by monitoring cell body position and axon morphology in larval stage L1 and adult animals using light microscopy. Additionally, staining with the fluorescent dye DiI, which fills defined subsets of head and tail neurons, was undertaken (as described by Hedgecock et al., 1985) on transgenic GFP reporter gene-expressing animals to compare green GFP fluorescence with red dye fluorescence using different microscope filter sets. The amphid neuron ADL and the phasmid neuron PHA showed overlapping GFP and dye staining; the relative positions of adjacent GFP reporter gene-expressing neurons as well as Nomarski optics were used to identify neighboring cells in the head and tail ganglia. The wild-type position of cells and the axon morphology of neurons have been described by Sulston et al. (1983) and White et al. (1986). Neuroanatomical defects (see Fig. 4) were identified by crossing the integrated *plin-11-DE-GFP* reporter array from wild-type into *lin-11(n389)* null mutant animals. VC neuroanatomical

defects (see Fig. 6) were identified with a *plin-11-B-GFP*; *rol-6* extrachromosomal array; four independent transgenic lines were examined and showed similar defects. Neuroanatomical defects were quantified by picking a random amount of transgenic, reporter gene-bearing animals (numbers given in the text) and were scored according to the categories described in the text.

Behavioral assays. Single worm thermotaxis assays were performed as described previously (Mori and Ohshima, 1995). Briefly, single animals were placed on 8 cm agar plates, which were then inverted, and a vial of frozen acetic acid (2.7 cm in diameter) was placed in the center on top of the agar plate to create a radial temperature gradient from 16 to 25°C. Animals were allowed to migrate in this radial temperature gradient for 1.5 hr, and their position and tracks on the agar plate were scored. For dauer formation assays, the synchronized brood of the respective genotypes was raised at different temperatures and scored for characteristic dauer features (Riddle and Albert, 1997). Because of the variability of dauer formation caused by subtle changes in the environment, comparisons of different genotypes were only made with assays that were done in parallel. Each assay was performed in triplicate.

RESULTS

Expression pattern of a *lin-11-GFP* reporter gene

The neural specific expression of many LIM homeobox genes in *C. elegans* (Freyd, 1991; Way and Chalfie, 1991; Hobert et al., 1997) (Hobert and Ruvkun, unpublished observations) prompted us to investigate the temporal and spatial control of expression of a *lin-11-GFP* reporter gene construct in the nervous system of *C. elegans*. A genomic DNA fragment bearing 6.5 kb of 5'-upstream sequences as well as the first five exons and four introns of *lin-11* was fused to GFP just upstream of the homeobox, transformed into *C. elegans*, and integrated into chromosomes to yield stable reporter gene expression. *lin-11-GFP* expression can be observed from late embryonic stages throughout larval and adult stages. Because of their characteristic position (Sulston et al., 1983), the *lin-11-GFP*-expressing cells were identified in early larval stages. At the L1 stage, *lin-11-GFP* expression is exclusively confined to neurons in the head ganglia and the lumbar ganglion in the tail (Fig. 2). The neurons that express *lin-11-GFP* in the head ganglion are the sensory neurons ADF and ADL and the interneurons AIZ and RIC (Fig. 3). The expression of *lin-11* in head sensory neurons correlates with the expression of the vertebrate *lin-11* homolog *Lhx1* in head sensory structures (Barnes et al., 1994). Weak *lin-11-GFP* expression can be observed in the interneuron AVG that sends a process along the ventral cord. A role for *lin-11* in AVG is suggested by the observation of Baumeister et al. (1996) that in *lin-11* mutant animals *unc-86* expression is aberrantly activated in AVG, suggesting that *lin-11* normally represses *unc-86* expression in AVG. The identity of another head neuron pair that exhibits *lin-11-GFP* expression could not be unambiguously determined, but because of its characteristic axonal morphology in the ventral cord (White et al., 1986), we tentatively assigned this pair of neurons as either the AVH or AVJ interneuron. The expression pattern of *lin-11-GFP* in the head ganglia is summarized in Figure 3.

Motor neurons within the vertebrate spinal cord express a specific set of LIM homeobox genes (Tsuchida et al., 1994). We find that *lin-11-GFP* is also expressed in motor neurons of the *C. elegans* ventral cord (Fig. 2). *lin-11-GFP* is activated in the six VC ventral cord motor neurons after they are generated during the L1 stage. Although their pattern of connectivity suggests a function in egg laying (White et al., 1986; Li and Chalfie, 1990), it has not been reported what kind of defects are caused by microsurgical removal of the VC ventral cord motor neurons. Adult *lin-11* null mutant animals display uncoordinated backward locomotion

(Freyd, 1991). It is unclear, however, whether this defect can be related to a function of *lin-11* in the VC motor neurons.

The *lin-11-GFP*-expressing tail neurons were identified as the PHA phasmid sensory neurons and the PVQ ventral cord interneurons, one of which (PVQL) extends a process on the left side of the ventral cord (Fig. 2). PVQ is a pioneer neuron of the ventral cord (Durbin, 1987) and required for correct axon fasciculation of follower neurons such as the HSN motor neuron (Garriga et al., 1993). HSN fasciculation also requires *lin-11* gene activity (Garriga et al., 1993). The expression of *lin-11* in PVQ but not HSN suggests that PVQ requires *lin-11* to provide fasciculation cues for HSN. It is interesting to note that all the neurons that express *lin-11* in the ventral cord are directly connected to other *lin-11*-expressing neurons; PHA forms synapses with AVG, PVQ, and AVH, and the VC neurons synapse onto PVQ (White et al., 1986). The functional significance of this observation is unclear, however (see Discussion).

Outside the nervous system, *lin-11-GFP* is expressed in cells of the developing vulva (Fig. 2), which correlates with the function of *lin-11* in these cells (Freyd et al., 1990). *lin-11* expression in the vulval precursor cells is dynamic and disappears once the vulva is formed. *lin-11-GFP* expression is also observed in the spermatheca (data not shown). A *lin-11-LacZ*-reporter gene fusion reveals a similar expression pattern (Freyd, 1991).

Temporal control of *lin-11* expression

The vertebrate *lin-11* homolog *Lhx1* has been shown to regulate embryonic inductions in pregastrulation stages (Taira et al., 1994; Shawlot and Behringer, 1995). In this regard, it is remarkable that no embryonic defects can be observed in *lin-11* mutants (Ferguson and Horvitz, 1985; Freyd et al., 1990). We corroborated the apparent absence of a role for *lin-11* in early embryogenesis by determining at which embryonic stage *lin-11-GFP* expression is activated. We found no detectable expression of *lin-11-GFP* in embryonic blastula and gastrula stages (Fig. 2; data not shown). *lin-11-GFP* expression is first observed in embryonic stages (~400 min) long after the beginning of gastrulation (at 100 min) (Sulston et al., 1983). The observed ~30 min delay between the onset of GFP protein expression and GFP fluorescence observed in *C. elegans* (G. Seydoux, personal communication) does not seem to be sufficient to account for the delay. To exclude any GFP maturation artifacts of our GFP reporter construct, we performed anti-GFP antibody staining on *lin-11-GFP*-expressing embryos and confirmed the postgastrulation embryonic expression of the *lin-11-GFP* reporter (data not shown). Moreover, a *lin-11-lacZ* fusion, the enzymatic activity of which is not subject to maturation, shows a similar onset of expression (Freyd, 1991). Thus, *lin-11* expression is activated significantly later in development than is that of its vertebrate homologs. The lack of early embryonic defects in a *lin-11* null mutant (Ferguson and Horvitz, 1985; Freyd et al., 1990) is consistent with this observation.

lin-11 expression is activated either significantly after the birth of the respective neuron (ADF, ADL, AIZ, AVG, AVH/AVJ, or VC) or at approximately the time the respective neuron is born (RIC). In the case of the VC motor neurons, this temporal control is particularly obvious; the VC motor neurons are formed in the L1 larval stage (Sulston et al., 1983) and extend their axonal projections along the ventral cord in L3 larval stages (Li and Chalfie, 1990). *lin-11-GFP* expression can first be observed in late larval stages once the VC motor neurons have sent out their axonal projections (Fig. 2). These observations demonstrate that activation of *lin-11* is a postmitotic event.

Figure 2. Expression pattern analysis of the *lin-11* gene. GFP fluorescence of different-staged animals carrying integrated *lin-11-GFP* reporter gene constructs are shown. The larval stages are indicated. The white arrow in the embryonic stages depicts an outgrowing axon from a neuronal cell body. Note that the GFP reporter construct does not contain the nuclear localization sequence of LIN-11; thus the axon and cell bodies fluoresce uniformly. Also note that because of differential planes of focus, not all neurons can be seen in every panel (e.g., in *A*, not all neurons in the lumbar ganglion are in the same plane of focus). *A, C, F*, Lateral view. *B, D, E*, Ventral and dorsal views. All animals shown (except those in *E* and *F*) carry an integrated *plin-11-ABCDE-GFP* reporter gene construct (see Fig. 4). Note that in the early L2 animal in *D*, the VC motor neurons are not matured (they have not sent out their axonal projections) (Li and Chalfie, 1990), so that in the ventral cord, only the *AVG*, *AVH/AVJ*, and *PVQ* interneurons stain. The white triangles in *D* point to the left (*PVQL*) and right (*AVG*, *AVH/AVJ*, and *PVQR*) ventral cord tracts. *E, F*, Transgenic *plin-11-B-GFP* animals, which display staining of the VC motor neurons and the vulva, are shown. The expression in the VC motor neurons begins at late larval stages after the postembryonic birth and axonal outgrowth of the VC neurons. Staining of tail neurons decreases in adulthood, whereas expression in the head neurons perdures. Expression in the vulval precursor cells is confined to the generation of the vulva during middle to late larval stages. Because the numbers of cells staining in these early larval stages does not appear to differ from the number of embryonically staining cells and because the onset of embryonic GFP staining correlates with the birth of these neurons, we conclude that the embryonic cells expressing *lin-11-GFP* are exclusively neurons. An essentially similar expression pattern can be observed using a lacZ-reporter gene fusion (Freyd, 1991). The expression of *lin-11* in the *AVG* interneuron is considerably weaker than that in the other head neurons.

The continuous expression of a transcription factor gene throughout the life of an animal suggests a role in maintenance of cellular function. In adult *C. elegans*, we find that *lin-11* expression is maintained at similar levels in all classes of head neurons throughout adulthood (Fig. 2). In contrast, expression in the tail neurons fades at late larval stages and cannot be observed in adult animals. These observations suggest a role for *lin-11* in the maintenance of neural function of head neurons. The perdurability of *lin-11-GFP* is not attributable to an artifactual stability of the reporter gene construct for several reasons. First, *lin-11-GFP* expression fades in tail neurons in postlarval stages, whereas it perdures in head neurons. Second, *lin-11-GFP* expression is rapidly turned off when *unc-86*, an upstream transcriptional regulator of *lin-11*, is conditionally turned off (J. Sze and G. Ruvkun,

unpublished observations). Third, a *ttx-3-GFP* fusion gene is similarly expressed throughout the life of the animal, and in this case the expression is dependent on *ttx-3(+)* activity, showing that the maintenance of GFP expression is an active process in this neuron (Hobert et al., 1997).

Defined *cis*-regulatory elements are responsible for *lin-11* gene expression

Deletion mutants of the original *lin-11-GFP* reporter gene define the control elements for *lin-11* expression (Fig. 4). A 3.5 kb region upstream of the putative ATG start codon (B element) mediates expression of *lin-11* in the developing vulva, the spermatheca, and the ventral cord motor neurons (Fig. 4). The *cis*-regulatory elements that mediate *lin-11* expression in neurons of the head and

neuron	neuron type	assigned function
ADF	sensory neuron	dauer formation & minor role in chemotaxis
ADL	sensory neuron	avoidance reaction to volatile repellents
AIZ	interneuron	thermotaxis, chemotaxis
RIC	interneuron	unknown function
AVG	interneuron	ventral cord pioneer neuron
AVH/AVJ	interneurons	unknown function

Figure 3. *lin-11*-expressing neurons in the head ganglion. The positions of the neurons in the anterior part of *C. elegans* are schematically shown. The drawing is adapted from Sulston et al. (1983) and White et al. (1986). The *lin-11*-expressing neurons are shaded, and their axonal projections are shown. Functions have been assigned to some of the *lin-11*-expressing neurons by laser ablation and subsequent behavioral assays. ADF, See Bargmann and Horvitz (1991a,b) and Schackwitz et al. (1996); ADL, see Troemel et al. (1995); AIZ, see Mori and Ohshima (1995) and Sze and Ruvkun (unpublished observations); and AVG, see Durbin (1987).

tail ganglion (DE elements) are localized to a region downstream to the putative translational start site (Fig. 4). Transgenes bearing an even shorter *cis*-regulatory element (E element) express only weakly in head neurons (Fig. 4). Interestingly, although the DE elements mediate initiation of *lin-11* expression in head neurons, they do not maintain *lin-11* expression throughout adulthood in all neurons (data not shown), suggesting that initiation and maintenance regulatory elements are separable.

It is remarkable that the regulatory elements responsible for driving *lin-11* expression in different classes of head neurons that are not closely related by lineage (Sulston et al., 1983) are confined to a small genomic 1.1 kb region downstream of the putative *lin-11* translational start site. The location of transcriptional regulatory elements within introns has been observed in various neuronally expressed genes, such as nestin in vertebrates (Zimmerman et al., 1994), *eyeless* in *Drosophila* (Quiring et al., 1994), or *unc-86* (Baumeister et al., 1996) and *ttx-3* (Hobert and Ruvkun, unpublished observations) in *C. elegans*. This observation underscores the compactness of the *C. elegans* genome, both in terms of gene structure and the structure of gene regulatory elements.

The paired-homeobox gene *Pax-6* is involved in neuronal patterning (Stoykova et al., 1996) and influences the combinatorial code of LIM homeobox genes that is thought to determine neuronal identity in the vertebrate brain and spinal cord (Tsuchida et al., 1994; Ericson et al., 1997; Osumi et al., 1997). Because the *C. elegans Pax-6* ortholog *vab-3* is also involved in head patterning (Chisholm and Horvitz, 1995), we determined whether expression of the *Lhx1/5* ortholog *lin-11* in either the head ganglion or in the ventral cord is under control of *vab-3*. However, the neuronally expressed *plin-11-DE-GFP* construct shows normal expression in animals with a loss-of-function *vab-3(e648)* mutation (data not shown).

LIN-11 does not autoregulate its expression

The only two other LIM homeobox genes in *C. elegans* for which loss-of-function phenotypes have been described are *mec-3* (Way and Chalfie, 1988) and *ttx-3* (Hobert et al., 1997). In both cases, the respective LIM homeobox gene is expressed throughout adulthood and is required for the maintenance of its own expression (Way and Chalfie, 1991; Hobert et al., 1997). We similarly

Figure 4. Definition of regulatory elements driving *lin-11* expression. The expression of various deletion derivatives of the original *plin-11-ABCDE-GFP* reporter construct was monitored in different tissues. The genomic structure of the *lin-11* gene plus the preceding gene (*ZC247.4*) is shown. The expression of the *plin-11-CDE-GFP* and *plin-11-DE-GFP* reporter constructs entirely recapitulates the neural expression of *plin-11-ABCDE-GFP*. Notably, some additional head neurons plus (in a few animals) some ventral and dorsal cord motor neurons weakly express *plin-11-DE-GFP*, arguing that the ABC elements contain negative regulatory elements. Note that the deletion derivatives *plin-11-CDE-GFP*, *plin-11-DE-GFP*, and *plin-11-E-GFP* do not contain the original translational start site (right-angle arrow) of the *lin-11* gene; these constructs presumably use either a potential downstream translational start site (stippled arrow) or the translational start site of the green fluorescent protein. *Vulva*, Cells of the developing vulva; *VC*, ventral cord motor neurons; and *Sper*, spermatheca.

observe continuous *lin-11-GFP* expression throughout adulthood, but we find that *lin-11* gene activity is not required for maintenance of its expression; no changes in the intensity of *lin-11-GFP* reporter gene expression were observed when comparing wild-type with *lin-11* null mutant animals (compare Figs. 2 and 5). This comparison was performed with an integrated transgenic array that was crossed from wild-type to mutant animals, excluding artifacts attributable to different transgenes. Factors other than *lin-11* must be required for maintenance of *lin-11* expression. In fact, J. Sze and G. Ruvkun have recently shown that continuous *unc-86* gene activity is required for maintenance of *lin-11* expression in the AIZ interneuron, the only overlap between the *unc-86* and *lin-11* expression patterns (Sze and Ruvkun, unpublished observations).

LIN-11-expressing neurons are generated but defective in *lin-11* null mutants

The *lin-11* gene was identified based on defects in vulval cell lineages (Ferguson and Horvitz, 1985). The neuronal expression pattern of *lin-11*, which we have described above, led us to investigate further what role *lin-11* might play in neurogenesis or neural function. We first wanted to determine whether *lin-11* gene activity is required for the generation of the *lin-11*-expressing neurons or whether *lin-11* affects neuronal development after the initial generation of neurons, as suggested by the temporal regulation of *lin-11* expression. We visualized the *lin-11*-expressing neurons in *lin-11(n389)* null mutant animals using a *lin-11-GFP* reporter gene construct. *lin-11(n389)* represents a null mutation

Figure 5. *lin-11*-expressing neurons are formed but display neuroanatomical defects in *lin-11* null mutants. To visualize the *lin-11*-expressing neurons in *lin-11(n389)* null mutants, we crossed an integrated *plin-11-DE-GFP* reporter gene into *lin-11(n389)* null mutant animals. Because of its structure (see Fig. 4), this reporter gene is not expected to have any *lin-11* gene activity. Young adult animals (aligned with anterior to the left and posterior to the right) with characteristic neuronal defects are shown. The reporter gene-expressing neurons in the head ganglion (most anterior-located cell bodies) are approximately similar in position and main axonal morphology to those in wild-type animals. Also, no obvious differences in fluorescence intensity can be observed (compare with Fig. 2). The transgenic animals carry the *rol-6* reporter gene as a marker, resulting in twisted worms. The white triangles (II, IV, V) point to the ventral cord. Note that a single animal can display several neuroanatomical defects at once or, alternatively, only a subset of them. There seems to be no linkage of the four classes of neuroanatomical defects shown in the table. I, V, The lower arrow (a) points to a process from the posteriorly displaced neuron AIZ that joins the ventral cord in an aberrant path toward the ventral cord. Normally, AIZ follows the amphid commissure to join the ventral cord (Fig. 3). I, II, The uppermost arrow (d) points to a posteriorly displaced neuron, most likely the AIZ interneuron. We define posterior displacement as the placement of the AIZ/RIC interneurons at locations significantly posterior to the posterior bulb of the pharynx. In wild type, the positions of AIZ, RIC, and other cells in the posterior lateral ganglia display some natural variability in their relative positions with respect to the posterior bulb of the pharynx; however, they always remain in close proximity to the pharyngeal bulb. In *lin-11* mutants, however, AIZ and RIC are displaced to a position significantly posterior to the posterior bulb of the pharynx. II, The posterior arrow marks a prematurely terminated process in the ventral cord. Note the fasciculation defects of processes in the ventral cord. III–V, Axons projecting posteriorly through a random path are shown (p).

in which the entire coding region of *lin-11* is deleted (Freyd et al., 1990). Because the *plin-11-ABCDE-GFP* reporter gene construct (Fig. 4) exhibits partial rescuing activity (data not shown), we used the *plin-11-DE-GFP* intronic promoter construct that has no *lin-11* gene activity and that entirely recapitulates the head neuronal expression pattern of *lin-11*. We crossed the chromosomally integrated *plin-11-DE-GFP* reporter gene array into *lin-11* null mutant animals. As judged by cell number and characteristic axon morphologies, we observed that all *lin-11*-expressing neurons are present in *lin-11* null mutant animals (Fig. 5), arguing that *lin-11* plays no major role in the generation of the *lin-11*-expressing neurons.

We do, however, observe subtle but readily detectable neuroanatomical defects in >60% of *lin-11* mutant animals. Represen-

tative examples are shown in Figure 5. Because the main axonal trajectories and the approximate cellular position are not severely disturbed, these types of defects are indicative of neural specification defects rather than lineage defects. The most obvious defects are (1) the presence of additional posteriorly directed processes that do not follow any specific path in the ventral or in the dorsal nerve cord and that terminate at random positions and (2) posteriorly displaced cell positions of some neurons, the axonal projections of which take unusual paths (Fig. 5). The identities of the posteriorly displaced neuronal cell bodies are most likely AIZ and RIC. It is possible that the posterior displacement of AIZ and RIC represents a migration defect of these neurons. The axons of the posteriorly displaced AIZ and RIC neurons often follow an aberrant path into the ventral cord (Fig.

<i>lin-11(n389)</i>		wildtype
normal neuroanatomy:	36 % (13/36 animals)	100 % (40/40)
neuroanatomical defects:	64 % (23/36 animals)	-
displaced interneuron cell body:	6/23	-
aberrant interneuron projection into ventral cord :	11/23	-
posteriorly directed process:	12/23	-
prematurely terminated process:	1/23	-

Figure 6. Motor neuron defects in *lin-11* null-mutant animals. The VC motor neurons were visualized using the *plin-11-B-GFP* reporter gene described in Figure 4. The VC motor neurons are the only neurons expressing this reporter, which allows us to visualize their anatomy independent of the ventral cord axonal processes of the *lin-11*-expressing head and tail neurons AVG, AVH/AVJ, and PVQ. The large white arrow points to one of the three VC motor neuron cell bodies shown; the small white arrows in the upper panel point to the fasciculated VC processes (up to six, depending on the position in the ventral cord) (White et al., 1986) in wild-type animals, whereas in the lower panels, the arrows point to the defasciculated VC processes in *lin-11* null mutants. Note that the animals are slightly twisted and lie upside down because of the *rol-6* marker, which allows us to obtain ventral views of the animals. The penetrance of the defect was determined by picking randomly >20 animals of each genotype that carried the transgenic marker *rol-6* and the GFP reporter construct as an extrachromosomal array and by scoring their defects. Similar defects were observed in four independent transgenic *lin-11* mutant lines.

5I,V, arrow a) that is distinct from the amphid commissure along which AIZ and RIC normally send their axonal projections into the ventral cord (see amphid commissure in Fig. 3). Examples of posteriorly directed processes are shown in Figure 5 (III–V, arrow p). Their origins are often hard to trace, but we observed cases in which they originate either from the posteriorly displaced neurons or, alternatively, from neurons in the more anterior head ganglion. These abnormalities are not observed in wild-type animals expressing the same, integrated *plin-11-DE-GFP* reporter gene construct. Occasionally, one of the processes in the ventral cord (AVG or AVH/AVJ) terminates prematurely in the *lin-11* mutant (Fig. 5II). Aberrant branching of posteriorly directed processes can also be observed (Fig. 5III,IV). Neuroanatomical defects of the ADF and ADL sensory neurons could not be observed; however, the axonal processes of ADF and ADL are difficult to follow because they fasciculate and run along with most other *lin-11*-expressing neurons in the amphid commissure and the nerve ring (Fig. 3).

Because *lin-11* is also expressed in some neurons of the ventral cord, we examined their fate in *lin-11* null mutant animals. The *plin-11-B-GFP* reporter gene construct (Fig. 4) was used to mon-

itor VC motor neuron anatomy in *lin-11* mutant animals. This reporter gene construct allows visualization of the VC motor neuron axonal projections without the confounding axonal processes that emanate from the *lin-11*-expressing head or tail neurons. We found that *lin-11* gene activity is not required for the VC motor neurons to be generated or to extend their processes along the ventral cord (Fig. 6). However, in >50% of the animals examined, the VC motor neurons displayed a defasciculated phenotype (Fig. 6). Normally, the six VC motor axons run parallel to one another and form multiple connections to each other as well as to other neurons in the ventral cord (White et al., 1986). Note that the head neurons that send processes along the ventral cord also show fasciculation defects (Fig. 5). The fasciculation defects we observe in the VC ventral cord motor neurons might be caused by a cell-autonomous requirement for *lin-11* in the VCs. Alternatively, these defects could be caused by defects in the *lin-11*-expressing AVG motor neuron, which is a pioneer neuron in the ventral cord (Durbin, 1987) and the laser ablation of which affects the bundling of neurons in the ventral cord, causing aberrant transfer and crossing of bundles (Durbin, 1987). In particular, several V- and D-type motor neurons display axonal defects if

Figure 7. *lin-11* null mutants display defects in thermotaxis. **A**, Schematic representation of the neural pathway subserving thermotaxis as revealed by laser ablation (Mori and Ohshima, 1995). Laser ablation of a neuron from one of the two pathways leads to uncontrolled activity of the other pathway, thus causing a thermophilic (laser ablation of AIZ) or cryophilic (laser ablation of AIY) phenotype. Sensory neurons are depicted by triangles; interneurons are indicated by hexagons. The connectivity of the neurons was delineated by White et al. (1986). **B**, *lin-11*(*n389*) null mutants exhibit a thermophilic phenotype. Animals were raised at 15°C and then tested on a thermal gradient. The mean values of three (Figure legend continues)

AVG is laser-ablated (Durbin, 1987). We addressed whether *lin-11* might have a role in AVG in guiding follower neurons by visualizing in *lin-11* null mutant animals several GABAergic V- and D-motor neurons in the ventral cord with an anti-GABA antibody (kindly provided by H. R. Horvitz; McIntire et al., 1993). No obvious axonal defects could be detected (data not shown). The expression of *lin-11* in the PHA phasmid neurons also prompted us to examine their neuroanatomy in *lin-11* mutant animals. Labeling the PHA sensory neuron with the fluorescent dye DiI, which fills amphid and phasmid sensory neurons (Hedgecock et al., 1985), we observed no obvious neuroanatomical defects of this pair of neurons in *lin-11* null mutant animals (data not shown).

***lin-11* mutants display thermotactic defects**

Some of the head neurons that express *lin-11* have been assigned functions by laser ablation and behavioral assays (Fig. 3). This type of analysis has revealed that the head interneuron AIZ plays a pivotal role in thermotaxis (Mori and Ohshima, 1995). Thermotaxis represents a learning paradigm in which the animal displays the capacity to sense and memorize its cultivation temperature so that it will migrate to its cultivation temperature when placed on a thermal gradient (Hedgecock and Russell, 1975). The thermotactic response relies on the output of two balanced and opposing temperature-processing pathways, one governed by the AIY and the other by the AIZ interneuron (Fig. 7A). Laser ablation of the AIY interneuron causes a cryophilic phenotype because of the unregulated activity of the other unaffected pathway, whereas laser ablation of the AIZ interneuron causes a thermophilic phenotype (Mori and Ohshima, 1995). Because *lin-11* is expressed in AIZ and because *lin-11* mutations cause AIZ neuroanatomical defects, we tested whether the absence of *lin-11* gene activity affects thermotaxis. Thermotaxis assays on *lin-11* null mutant animals revealed a thermophilic phenotype (Fig. 7B), which is similar to laser ablation of AIZ. The assignment of thermotactic function with AIZ is underscored by the similar thermophilic phenotype of *unc-86* mutant animals (Fig. 7B) (Mori and Ohshima, 1995), which do not generate the AIZ interneuron because of a cell lineage defect (Finney and Ruvkun, 1990). Because AIZ is formed but apparently not functional in *lin-11* mutant animals (Fig. 5), we conclude that *lin-11* is required for the functional specification of AIZ.

Another temperature-controlled behavior is the execution of the dauer developmental program (Golden and Riddle, 1984). Specific sensory inputs that are indicative of harsh environmental conditions, such as low amounts of food, crowding, and high temperature, are processed by the *C. elegans* nervous system to cause arrest of *C. elegans* larvae at the dauer stage (Riddle and Albert, 1997). Considering the thermotactic defect of *lin-11* (Fig. 7B), we asked whether *lin-11* loss of function also affects temperature modulation of dauer arrest. We observed a small but significant suppression of *daf-7*-induced dauer for-

mation at 25°C (for *daf-7(e1372); lin-11(n389)*, $15 \pm 4\%$ suppression; $n = 928$; for *daf-7(e1372)*, 0% suppression; $n > 100$). However, because a variety of unrelated genes also display weak suppression of dauer formation, we cannot conclude that *lin-11* affects dauer formation specifically through the thermosensory neural circuit.

DISCUSSION

Specification of a neural network by two LIM homeobox genes

C. elegans thermotactic behavior represents one of the few animal behaviors the neuronal components of which have been well defined, revealing the existence of a specific neural regulatory network that subserves this behavior (Mori and Ohshima, 1995). The organization of this network into two parallel, warm- and cold-processing thermoregulatory pathways is remarkably similar to that of thermocontrol in vertebrates (Boulant and Dean, 1986). The molecular basis for the generation and function of this *C. elegans* pathway may therefore be related to the mechanism by which vertebrate thermoregulation is achieved.

We have shown previously that the AIY interneuron that is necessary for thermophilic responses requires a LIM homeobox gene, *ttx-3*, for its functional specification (Hobert et al., 1997). In the case of *ttx-3*, it is expressed only in AIY, and the lack of *ttx-3* gene activity affects AIY structure and the thermoregulatory function assigned to AIY by laser ablation. Our assignment of *lin-11* function in thermoregulation to the AIZ interneuron involves a similar argument. (1) A *lin-11-GFP* fusion gene is expressed in AIZ, (2) the neuroanatomy of AIZ is affected in a *lin-11* null mutant, and (3) the thermoregulatory function assigned to AIZ by laser ablation is affected similarly in a *lin-11* null mutant. Although we cannot exclude the possibility that the thermotactic defect of *lin-11* mutants is because of defects in other *lin-11*-expressing neurons, we consider this unlikely for the following reasons. First, none of the other *lin-11*-expressing neurons has been implicated in thermotaxis (Mori and Ohshima, 1995); second, *unc-86* causes similar thermotactic defects (Fig. 7B), and the only overlap between *unc-86* and *lin-11* expression in postmitotic head neurons is in AIZ. Thus we suggest that the *lin-11* LIM homeobox gene acts in the AIZ interneuron that is functionally antagonistic to the AIY *ttx-3*-expressing interneuron (Fig. 7A).

The entry of *C. elegans* into the developmentally arrested dauer stage is also under thermosensory control (Golden and Riddle, 1984; Hobert et al., 1997). Our finding that *lin-11* affects dauer formation could be explained with a defect in thermoregulation of dauer formation. This hypothesis is consistent with the expression of *lin-11-GFP* in the thermoregulatory AIZ interneuron. However, the effect of *lin-11* on dauer formation is considerably weaker than the effect of *ttx-3* on dauer formation (Hobert et al., 1997) and could be an effect of *lin-11* unrelated to thermocontrol of dauer formation [e.g., *lin-11* could affect sensory function of the ADF neuron, which controls the dauer program (Bargmann

representative assays are shown; in each assay, single animals (using 10–15 *lin-11*, 9–15 control, and 5–15 *unc-86* animals) from each different genotype were tested in parallel. The tracking of animals in the thermal gradient was classified into three categories: animals that have learned to remember the 15°C cultivation temperature and move to and track within the 16°C region of the thermal gradient (*gray bars*), animals that move irrespective of their cultivation temperature to higher temperatures (*red bars*; thermophilic), and animals that track within the 16°C region of the gradient but occasionally also outside this region (*hatched red bars*). *lin-11(n389)* hermaphrodites display slightly uncoordinated movement, which hampers the thermotaxis assay. Because *lin-11(n389)* males do not exhibit this uncoordinated phenotype, *lin-11(n389)*, *him-5(e1467)* males were assayed for thermotactic behavior. Consequently, *him-5(e1467)* males were used as control animals. *unc-86(n846)* animals lack AIZ because of a cell lineage defect (Finney and Ruvkun, 1990) and thus display a strong thermophilic phenotype (Mori and Ohshima, 1995).

and Horvitz, 1991a)]. Thus, although *ttx-3* and *lin-11* are clearly both required for thermotactic behavior, it remains an open issue as to how much the neural and genetic components that couple thermosensory information to a motor output are the same as the neural components that couple to dauer development. Perhaps the major output to neuroendocrine control is via AIY and not AIZ.

The major thermoregulatory organ of vertebrates, the hypothalamus, contains distinguishable warm- and cold-sensing temperature-processing units (Boulant and Dean, 1986) that may be homologous to the antagonistic high and low temperature-sensing pathways of the *C. elegans* thermotactic response pathway (Fig. 7A). *lin-11* and *ttx-3* in *C. elegans*, and their homologs in mammals, may thus mediate the development of two components of this phylogenetically conserved thermal-processing network. In support of this hypothesis, the vertebrate *ttx-3* homolog *Lhx2* and the *lin-11* homologs *Lhx1* and *Lhx5* are expressed in the diencephalon, which gives rise to the thermoregulatory hypothalamus (Fuji et al., 1994; Porter et al., 1997; Sheng et al., 1997).

The AIY and AIZ neurons are formed in animals bearing null mutations in *ttx-3* and *lin-11*, respectively, but these neurons show subtle anatomical defects suggesting that they fail to execute late events in neurogenesis. For example, TTX-3 and LIN-11 may regulate genes that are directly involved in axonal pathfinding, so that the patterns of neuroanatomical defects observed in both *ttx-3* and *lin-11* mutant animals can be explained on the basis of direct disturbances in axonal pathfinding. Alternatively, these genes may regulate the expression of genes that mediate synaptic connectivity or synaptic activity, and these neural defects may be secondary consequences of the absence of synaptic signaling. Synaptic connectivity-dependent sprouting of additional processes has been observed in other systems (e.g., Henderson et al., 1983).

The expression and function of two genes from the same gene family in two parallel neural pathways provokes the question as to whether *ttx-3* and *lin-11* are differentially instructive to determine specific AIY and AIZ features, respectively. Alternatively, they could act permissively to regulate the same set of downstream target genes in order to determine features that are common to AIY and AIZ [e.g., their connection to the thermotactic RIA interneuron, which is a postsynaptic target of both AIY and AIZ and required for thermotactic behavior (Mori and Ohshima, 1995)]. In *Drosophila*, the paired-class homeobox genes *prd*, *gsh*, and *gsbn* are differentially expressed but can functionally substitute for one another (Li and Noll, 1994). Their divergent roles may have simply evolved by changes in deployment. The compactness and modularity of the *cis*-regulatory elements for *lin-11* (Fig. 4) and *ttx-3* (Hobert and Ruvkun, unpublished observations) would facilitate the evolution of distinct developmental function by acquisition of distinct regulatory elements. In summary, our data suggest that distinct LIM homeobox genes may determine the features of functionally related neurons of neural circuits that generate specific behaviors.

LIN-11 function in the ventral cord

We find that *lin-11*-expressing motor neurons in the ventral cord are formed in *lin-11* null mutant animals but display neuroanatomical defects. There are six VC motor neurons in the ventral cord, the axonal projections of which fasciculate and run in parallel in the ventral cord to form chemical and electrical synapses with each other and with other neurons and target muscles. We find that *lin-11* gene activity is not required for the generation or the axonal outgrowth of these neurons but required for them

to fasciculate correctly. It is possible that LIN-11 regulates downstream target genes that are required for homophilic interaction of the VC motor neurons; alternatively, these downstream target genes might be required for correct fasciculation with other neurons in the ventral cord. It is also possible that the VC motor neuron fasciculation defects are caused by absence of *lin-11* function in the AVG neuron, which is a pioneer in the ventral cord (Durbin, 1987); however, because we do not observe defects in other ventral cord motor neurons that require AVG to fasciculate, such as the V- and D-type motor neurons (Durbin, 1987), we consider this possibility less likely.

The HSN motor neuron requires the PVQ ventral cord pioneer to fasciculate correctly (Garriga et al., 1993). Moreover, Garriga et al. (1993) have shown that *lin-11* gene activity is required for proper HSN fasciculation. Our identification of PVQ, but not HSN, as one of the *lin-11*-expressing neurons is consistent with a hypothesis in which *lin-11* acts in PVQ to affect HSN morphology. The function of *lin-11* in the VC motor neurons and PVQ neurons might thus be similar in that LIN-11 could be required to provide cues for selective fasciculation of neurons in the ventral cord. For example, LIN-11 could regulate the expression of adhesive cell surface receptors that mediate fasciculation between VC neurons and between PVQ pioneer and HSN follower axons. It is also intriguing to note that all the *lin-11*-expressing neurons in the ventral cord have at least one synaptic partner that also expresses *lin-11*. VC synapses with PVQ and HSN; PHA synapses with AVG, AVH, and PVQ; and PVQ synapses with VC and HSN. Thus, another hypothesis posits that *lin-11* is required to determine synaptic connectivity and that the fasciculation defects of the VC and HSN neurons are a secondary consequence of missing synaptic inputs. Although we do not observe fasciculation defects of the *lin-11*-expressing head neurons in *lin-11* mutants, the process outgrowth and migration defects of the head neurons could also be explained by misregulation of cell surface molecules required for correct axonal guidance or synaptic activity.

The vertebrate *lin-11* ortholog *Lhx1* is also expressed in motor neurons of the spinal cord. *Lhx1* null mutant mice die because of early embryonic defects (Shawlot and Behringer, 1995); thus the importance of *Lhx1* in motor neurons could not be addressed to date. Regarding the function of *lin-11*, it is conceivable that *Lhx1* might act in a similar manner in determining axonal morphology of spinal cord motor neurons.

A conserved neural function for LIM homeobox genes

Loss-of-function studies in *C. elegans* and *Drosophila* have shown that LIM homeobox genes display a postmitotic function late in neural development. *C. elegans mec-3* acts in touch cell receptor specification (Way and Chalfie, 1988, 1991); *ttx-3* (Hobert et al., 1997) and *lin-11* as shown here act in thermotactic interneuron specification. All three genes continue to be expressed at high levels throughout adulthood. Two recently identified *C. elegans* LIM homeobox genes are also expressed in a very restricted subset of postmitotic neurons, and their expression is also maintained throughout adulthood (Hobert and Ruvkun, unpublished observations). Axonal pathfinding defects have been described in *Drosophila* mutants of the LIM homeobox genes *apterous* and *islet* (Lundgren et al., 1995; Thor and Thomas, 1997); *Drosophila islet* also controls neurotransmitter identity (Thor and Thomas, 1997). Also, vertebrate LIM homeobox genes from all LIM homeodomain subclasses (Fig. 1) have been shown to be expressed continuously throughout adulthood, mostly in the brain (Thor et al.,

1991; Taira et al., 1992; Xu et al., 1993; Barnes et al., 1994; Fuji et al., 1994; Gong et al., 1995; Sheng et al., 1997). Taken together, these observations suggest a common theme for the function of LIM homeobox genes late in neural differentiation, potentially in the establishment of features required for synaptic signaling as well as for the maintenance of the differentiated state of neurons. Additional and relatively specialized functions of LIM homeobox genes, such as *lin-11* function in vulva formation (Ferguson and Horvitz, 1985; Freyd et al., 1990), *apterous* function in wing development (Cohen et al., 1992), *Lhx3* function in pituitary development (Sheng et al., 1996), or *Lim1* function in organizer activity (Taira et al., 1994; Shawlot and Behringer, 1995), might have been co-opted by specific phyla at different stages of evolution.

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