

The *C. elegans* homeodomain gene *unc-42* regulates chemosensory and glutamate receptor expression

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Accepted 22 February; published on WWW 19 April 1999

SUMMARY

Genes that specify cell fate can influence multiple aspects of neuronal differentiation, including axon guidance, target selection and synapse formation. Mutations in the *unc-42* gene disrupt axon guidance along the *C. elegans* ventral nerve cord and cause distinct functional defects in sensory-locomotory neural circuits. Here we show that *unc-42* encodes a novel homeodomain protein that specifies the fate of three classes of neurons in the *Caenorhabditis elegans* nervous system: the ASH polymodal sensory neurons, the AVA, AVD and AVE interneurons that mediate repulsive sensory stimuli to the nematode head and anterior body, and a subset of motor neurons that innervate head and body-wall muscles. *unc-42* is required for the expression of cell-surface

receptors that are essential for the mature function of these neurons. In mutant animals, the ASH sensory neurons fail to express SRA-6 and SRB-6, putative chemosensory receptors. The AVA, AVD and AVE interneurons and RME and RMD motor neurons of *unc-42* mutants similarly fail to express the GLR-1 glutamate receptor. These results show that *unc-42* performs an essential role in defining neuron identity and contributes to the establishment of neural circuits in *C. elegans* by regulating the transcription of glutamate and chemosensory receptor genes.

Key words: *unc-42*, Homeodomain, Mechanosensory, Axon pathfinding, *Caenorhabditis elegans*

INTRODUCTION

Neural development requires the generation of a diverse population of neuron types distinguished by morphology, connectivity patterns and the expression of distinct combinations of neurotransmitters, receptors and ion channels. Even the relatively simple nervous system of the *C. elegans* hermaphrodite is made up of 118 different classes of neurons (White et al., 1986). Cell fate specification in the nervous system is complex, requiring the combinatorial action of multiple transcriptional regulators (reviewed in Bang and Goulding, 1996; Ruvkun, 1997; Sengupta and Bargmann, 1996; Tanabe and Jessell, 1996; Doe and Skeath, 1996). Extensive studies in invertebrates and vertebrates have identified genes that act at early stages of neuronal fate determination and patterning of the nervous system, but the mechanisms that generate specific neuronal subtypes are still poorly understood.

One important group of transcriptional regulators defined by genetic mutations are the paired-like homeobox proteins. Members of this class contain homeodomains closely related to the homeodomains of the Prd/Pax gene family (Frigerio et al., 1986), but lack the paired domain. The neuronal paired-like homeoproteins regulate particular neuronal traits in subsets of

neurons, and thus are candidates to act downstream of transcriptional regulators that play more general roles in determining whether a cell assumes a neuronal fate. The *C. elegans* UNC-4 protein, for example, regulates synaptic specificity of one class of motor neuron (Miller et al., 1992; White et al., 1992). The VA motor neurons of *unc-4* mutants receive inappropriate synaptic inputs from interneurons that normally synapse onto another class of motor neuron. Yet, other aspects of VA differentiation appear normal, including their ability to make appropriate neuromuscular synapses. Phox2A, a mouse homeobox protein of this class, is required for specific aspects of noradrenergic cell fate, including neurotransmitter synthesis and expression of Ret, the receptor for glial derived neurotrophic factor (GDNF) (Morin et al., 1997).

In this paper, we describe the characterization of *unc-42*. We show that *unc-42* encodes a novel homeodomain protein that is related to UNC-4 and other *C. elegans* paired-like homeodomain proteins, but is expressed by a different subset of neurons. Cells that require *unc-42* function include the AVA, AVD and AVE interneurons that control backward movement and the polymodal ASH sensory neurons that mediate aversive responses to mechanical stimulation, chemorepellents and high osmolarity. Our analysis indicates that *unc-42* mutations eliminate expression of two putative chemoreceptors, SRA-6

and SRB-6, by the ASH neurons, while other general neuronal traits are not affected. We show that *unc-42* mutants also fail to express the GLR-1 glutamate receptor in many of the interneurons and motor neurons that form the neural circuitry for nose touch. Our results demonstrate that the neural circuits for forward and backward motion in *C. elegans* are regulated by separate developmental programs and reveal a potential role for UNC-42 as a direct transcriptional regulator of glutamate receptor and chemosensory receptor expression.

MATERIALS AND METHODS

Strains and genetics

Strains were grown at 20°C and maintained as described by Brenner (1974). In addition to the standard wild-type strain (N2), strains with the following mutations were used in this work:

LGII: *gmls13* [*srb-6-gfp* + *rol-6*] (N. Hawkins, personal communication; Troemel et al., 1995).

LGIII: *ncl-1(e1865)*.

LGIV: *unc-42(e270)*, *unc-42(e419)*, *unc-42(e623)*, *unc-42(gm23)*; *smg-4(ma116)*.

LGX: *dpy-11(e6)*; *nuls1* [*glr-1-gfp* + *lin-15*] *lin-15(n765ts)* (Hart et al., 1995).

Chromosomal aberrations: *eT1(III,V)* (Rosenbluth and Baillie, 1981); *sDf29(V)* (Rosenbluth et al., 1985).

Extrachromosomal arrays: *gmEx104* [pRB18::*rol-6(dm)*], *gmEx186*[pRB22 + *rol-6(dm)*], *gmEx71* [*sra-6-gfp* + *rol-6(dm)*] (Wightman et al., 1997; Troemel et al., 1995).

Free chromosomal duplication: *ctDp11* (Hunter and Wood, 1992).

Mosaic analysis

The method of Herman (1984) was used for genetic mosaic analysis. We constructed a strain that was mutant for *unc-42(e270)* and the cell-autonomous marker *ncl-1* (Kenyon, 1986; Hedgecock and Herman, 1995), and carried *ctDp11*, a free chromosomal duplication containing wild-type copies of both genes (Hunter and Wood, 1992). Cells that lose the duplication and are mutant for *ncl-1* have enlarged nucleoli and can be identified by Nomarski optics. To analyze the defects in locomotion and the response to light body touch, we identified Unc non-Mec and Mec non-Unc F₁ mosaic hermaphrodites and determined the point in the cell lineage where loss of the duplication occurred.

Analysis of *unc-42* heterozygotes and *sDf29* heterozygotes

To determine if *unc-42* heterozygotes exhibit HSN pathfinding defects, wild-type males were mated to *dpy-6(e11)*; *unc-42* double mutants. HSN axon morphology was examined in F₁ cross progeny and *dpy-6(e11)*+ control animals by anti-serotonin staining (Garriga et al., 1993a). Self-progeny exhibit the short, dumpy phenotype of *dpy-6* and are easily distinguished from *unc-42*+ heterozygotes that have a wild-type body shape. To examine pathfinding defects in deficiency hemizygotes, we analyzed animals that contained *sDf29*, a deletion chromosome that uncovers the *unc-42* gene, balanced by *eT1* (*dpy-18(e364)III/eT1*; *unc-46(e177)sDf29 V/eT1*). *eT1* is a reciprocal translocation of chromosomes II and V that breaks within the *unc-36* gene, generating viable translocation homozygotes that are Unc. To examine HSN axon morphology in *sDf29*+ animals, 200 non-Unc-36 hermaphrodites were transferred to a separate plate and stained with antiserum to serotonin (Garriga et al., 1993a).

Cloning *unc-42*

unc-42 mapped genetically to the right of *her-1* on chromosome V. *her-1* is located on cosmid C12G5 on the *C. elegans* physical map (Trent

et al., 1991). Both *her-1* and *unc-42* are removed by the small chromosomal deletion, *sDf29*. The right end-point of *sDf29* was mapped to cosmid T07C12 by RFLP analysis of wild-type and *sDf29* genomic DNA. Rescue of the Unc and Mec phenotypes and ventral cord pathfinding defects of *unc-42* was obtained when mutant worms were injected with either of two YACs that bridge the region, Y1D1 and Y42F5 (Coulson et al., 1986). All germline transformation experiments were conducted as described by Mello et al. (1991). Y42F5 and Y1D1 YAC DNA were purified by pulsed-field gel electrophoresis, treated with β-agarase (USB) and concentrated in Centricon-30 microconcentrators (Amicon). *unc-42(e270)* *smg-4(ma116)* hermaphrodites were injected with 5 μg/ml of YAC DNA plus 20 μg/ml of *rol-6(su1006dm)* DNA (pRF4) as a dominant selectable marker.

Y42F5 was used to construct a Lambda FixII phage library. Genomic DNA from yeast carrying the Y42F5 YAC was partially digested with *Sau3AI* and ligated into the Lambda FIXII vector using the Lambda FIXII/*XhoI* Vector system from Stratagene. 60,000 plaques from the Y42F5 Lambda FixII library were screened with three DNA probes generated by random priming (Feinberg and Vogenstein, 1983). Probes were made from purified Y1D1 and Y42F5 YAC DNA and B15.1, a circular deletion derivative of Y42F5 generated as described by Miller et al. (1993) and isolated by the method of Devenish and Newlon (1982). DNA was prepared from 59 λ clones that hybridized with all three probes and tested in pools of 5-6 for rescuing activity. *unc-42(e270)* hermaphrodites were injected with DNA from the Lambda FIXII phage clones at concentrations of 50 μg/ml with 100 μg/ml of *rol-6(su1006)* DNA. A single 16.8 kb phage clone, λRB162, rescued the *unc-42* mutant phenotypes. Subclones of λRB162 were constructed in pBluescript KS⁻ and used to identify a minimal rescuing region of 7.8 kb.

Isolation, PCR and cloning of *unc-42* cDNAs

A 4.2 kb *SaII-EagI* genomic fragment from the *unc-42* minimal rescuing region was used to screen 500,000 plaques of a λgt10 *C. elegans* cDNA library (gift of L. Miller and B. Meyer). Three cDNA clones were identified; all contained inserts of approximately 1.2 kb and showed the same pattern of restriction sites. Inserts were amplified by PCR using primers to λgt10 flanking sequence (5'CGGGATCCCCGTCAGCCTGGTTAAGTCCAAG3' and 5'CGGGATCCCCGTTATGAGTATTCTCCAGG3') and ligated into pBluescript KS⁻. Two additional cDNA clones were generated by RT-PCR (primers: 5'GGAAGCTAGCAATGAGCGACAATCTGC3' and 5'GGGGTACCACTAGATTGAAGGAGG3'). Complete double-strand sequence obtained for the cDNAs indicated that they were identical.

Northern blot hybridization

Poly(A)⁺ RNA from embryonic and mixed stage cultures of *C. elegans* was prepared as described by Garriga et al. (1993b). Electrophoresis, blotting and probing of the RNA was performed by standard molecular biology techniques (Sambrook, 1989). An *unc-42* cDNA clone was used to produce an RNA probe that hybridizes to the C-terminal two-thirds of the *unc-42* transcript.

Sequence of genomic and *unc-42* mutant DNA

C. elegans genomic sequence for the *unc-42* region was obtained using the transposon-mediated method of Strathmann et al. (1991) and assembled using AssemblyLIGN software (Kodak). Double-strand sequence was generated for overlapping subclones of λRB162 that constituted the minimal rescuing region of *unc-42* in germline transformation experiments. This region was sequenced subsequently by the *C. elegans* genome project. *unc-42* corresponds, in part, to the predicted gene F58E6.10.

For analysis of *unc-42* mutant alleles, genomic DNA from mutant strains was amplified by single-worm PCR and ligated into pBluescript KS⁻. For each mutant strain, double-strand sequence was obtained for two or three subclones constructed from DNA amplified in independent PCR reactions. Single mutant worms were prepared

as described by Williams et al. (1992). DNA was amplified using the Expand Long Template PCR System (Boehringer Mannheim).

***unc-42-gfp* reporter gene construction**

The pRB18 construct contains 2.6 kb of upstream *unc-42* genomic sequence fused to the GFP coding sequence (Chalfie et al., 1994) at the N terminus of *unc-42*. To construct pRB18, a new *XbaI* site was introduced by PCR at the initiation codon of *unc-42*. An 80 bp sequence generated by digesting the PCR product with *SalI* and *XbaI* was co-ligated with a 2.5 kb *HindIII-SalI* genomic fragment into the *HindIII-XbaI* site of the GFP expression vector pPD95.77 (Gift of A. Fire). pRB22 contains 7.5 kb of *unc-42* genomic sequence and encodes a full-length UNC-42 protein with a GFP tag at the C terminus. pRB22 was generated by reconstructing the *unc-42* genomic region in pRB18. A new *XbaI* site was inserted at the 3' end of the *unc-42* coding sequence by PCR.

pRB18 and pRB20 (*unc-42-gfp*) were injected at 100 µg/ml with 100 µg/ml of pRF4 into wild-type hermaphrodites. F₁ transformants that carried the injected DNA as an extrachromosomal array were identified by the dominant roller phenotype conferred by coinjected *rol-6(su1006)* DNA. In transformation rescue experiments, F₂ roller lines were tested for rescue of the *unc-42* Unc and Mec behavioral phenotypes and assayed for defects in HSN pathfinding by anti-serotonin staining (Garriga et al., 1993a).

UNC-42 antiserum

A His-tagged UNC-42 fusion gene was constructed by inserting the C-terminal two-thirds of the *unc-42* cDNA into the pRSET-B vector beginning with the *BamHI* site in exon 2 (Invitrogen). A fusion protein of the predicted size of 31 kDa was purified on nickel agarose and injected subcutaneously into mice and rabbits (Harlow and Lane, 1988). Antibody experiments used either polyclonal ascites from mice or polyclonal antisera from rabbits purified against fusion protein immobilized on nitrocellulose strips.

Immunofluorescence histochemistry, dye loading and microscopy

For antibody staining, strains were grown at 20°C unless stated otherwise. Embryos were prepared by treating gravid hermaphrodites and freshly laid eggs with 5 ml of hypochlorite solution (0.7 M NaOH, and 4.4% NaOCl) for 10-15 minutes. Embryos were fixed and permeabilized as described by Guenther and Garriga (1996). Fixed embryos were incubated at room temperature overnight in primary antibody solution [1:10 dilution of mouse anti-UNC-42 antiserum and 1:3000 dilution of rabbit anti-UNC-86 (Finney and Ruvkun, 1990) in PBST-A (1× PBS, 1% BSA, 0.5% Triton X-100, 0.05% Na₃, 1 mM EDTA)]. For double staining embryos that expressed GFP fusion proteins, GFP was detected using a 1:500 dilution of rabbit anti-GFP (Clontech).

First larval stage (L1) worms were obtained as described by Guenther and Garriga (1996). To detect UNC-42 and MEC-7 in larvae and adults, animals were washed, fixed, permeabilized and stained by the method of Finney and Ruvkun (1990). Fixed worms were incubated overnight in primary antibody solution [1:10 dilution of mouse or rabbit anti-UNC-42 and 1:100 dilution of anti-α-tubulin (Piperno and Fuller, 1985) in PBST-A]. For double staining larvae and adults with DAPI and anti-serum to GFP, worms were fixed and permeabilized as described by Garriga et al. (1993a). GFP was detected using a 1:500 dilution of rabbit anti-GFP (Clontech).

For application of secondary antibodies, all worms and embryos were washed 3× and incubated for 3 hours or overnight at room temperature in secondary antibody solution (1:400 dilution of Cy-3-conjugated donkey anti-mouse antiserum (Jackson Immunoresearch) and/or 1:300 dilution of FITC-conjugated goat anti-rabbit antiserum (Cappel) in PBST-A).

Sensory neurons were visualized in larvae and adult hermaphrodites with the fluorescent dye DiI as described by Garriga et al. (1993b).

Worms prepared for immunofluorescence were viewed and photographed with Ektachrome T160 film using a Zeiss microscope and filters #487910 and #487915. Photographs were scanned into a computer graphics file with a Nikon scanner and annotated with the Adobe Photoshop graphics program.

Behavioral tests

Mechanosensory function for light body touch was assayed by the method of Chalfie and Sulston (1981), and nose touch was determined as described by Kaplan and Horvitz (1993).

RESULTS

Mutations in *unc-42* alter locomotion and disrupt multiple sensori-locomotor neural circuits

The first three *unc-42* alleles were identified based on the uncoordinated (Unc) phenotype conferred by the mutations (Brenner, 1974). Although mutant worms can move forward or backward spontaneously, this movement is slow and irregular. Mutant worms tend to kink ventrally and their head movement is restricted. These defects are likely to be the result of neural defects because muscle development and morphology are normal in *unc-42* mutants (Waterston and Francis, 1985).

In additional assays for behavioral defects, we found that *unc-42* mutants also exhibited severe defects in response to mechanical stimuli to the nematode head and anterior body. *unc-42* mutants exhibited a distinctive mechanosensory (Mec) response to light touch along the body: mutant animals failed to back up when stroked along anterior body regions, but moved forward normally when touched along the posterior body (Table 1). The response to touch at the tip of the nose (Not) was also severely reduced in the mutants. Only 8% of *unc-42 (e419)* animals responded to nose touch, compared to 90% for wild-type worms (Table 1).

Nose touch and body touch are mediated through separate neural circuits defined by anatomy (White et al., 1986), cell killing experiments (Chalfie et al., 1985) and genetic analysis (Hart et al., 1995; Maricq et al., 1995). Wild-type animals respond to light strokes to the anterior part of the body by

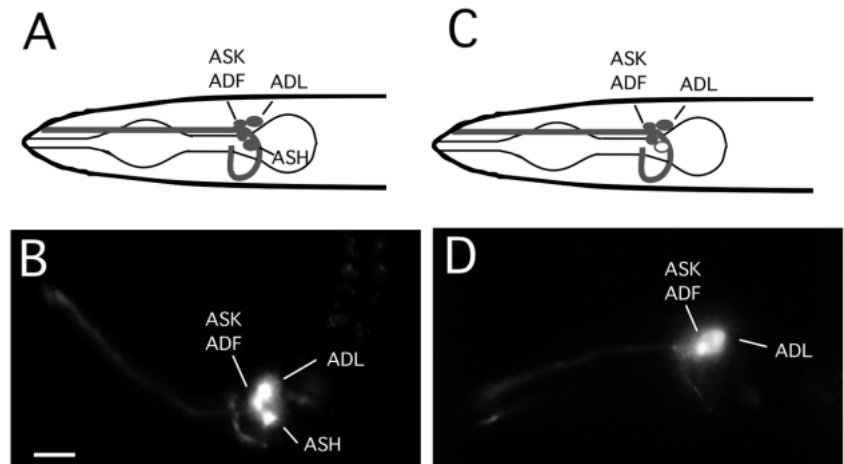
Table 1. Mechanosensory defects in *unc-42* mutants

Strain	% response*		
	Anterior body touch	Posterior body touch	Nose touch
<i>wild-type</i>	100	98±2	90±5.9
<i>unc-42(e419)</i>	2±2	96±2.8	8±3.8
<i>unc-42(e270)</i>	0	94±3.4	10±4.3
<i>glr-1(n2461)</i>	98±2	96±2.8	12±4.6

unc-42 mutant adult hermaphrodites were tested for response to light touch along the anterior and posterior body (Mec) and touch to the tip of the nose (Not). Wild-type and *glr-1(n2461)* mutant adult hermaphrodites were tested as controls.

*50 animals for each strain were scored for anterior and posterior body touch, and nose touch. Light touch to the body was assayed by lightly stroking the side of the head or tail as described by Chalfie et al. (1985). Each animal was tested at least three times. Animals were scored as responding to anterior touch if they moved backward when touched along the head or anterior. Posterior touch was scored as normal if the animals moved forward or stopped backward movement when touched along the tail or posterior body. Nose touch was tested as described by Kaplan & Horvitz (1993). Animals were tested at least five times and were scored positive for nose touch if they stopped or moved backward in response to stimulus at the tip of the nose. Errors indicate the s.e.m.

Fig. 1. *srb-6-gfp* transgene expression in *unc-42* mutant animals. The ASH sensory neurons in *unc-42* mutants fail to express *gmlS13*, an integrated *srb-6-gfp* transgene. (A,C) Diagram of the pattern of *srb-6-gfp* expression in the anterior of (A) wild-type and (C) *unc-42* larvae. Circles depict the cell bodies on the left side. Solid circles represent the sensory neurons that express *srb-6-gfp*; the open circle represents ASH in an *unc-42* mutant, which fails to express *srb-6-gfp*. (B,D) Fluorescence photomicrographs of left lateral views of (B) wild-type and (D) *unc-42(e419)* larvae showing expression of the *srb-6-gfp* transgene. Scale bar, 10 μ m.



backing up and to touch along the posterior body by moving forward. The ALMR, ALML and AVM neurons sense light touch along the anterior body, and the PLMR and PLML neurons sense touch along the posterior body. Anterior body touch requires the function of the AVA and AVD interneurons, which innervate type-A motor neurons, to drive backward movement, while posterior body touch requires the AVB and PVC interneurons, which innervate type-B motor neurons, to drive forward movement (Chalfie et al., 1985; White et al., 1986). The response to anterior body touch is dependent primarily on the AVD interneurons, probably via gap junctions with the ALM and AVM sensory neurons (Chalfie et al., 1985).

Avoidance to touch at the tip of the nose is detected primarily by the sensory neurons ASH and FLP (Kaplan and Horvitz, 1993). The ASH neurons also sense high osmolarity and volatile repellents (Bargmann et al., 1990; Troemel et al., 1995), and may be analogous to vertebrate nociceptors that detect several pain modalities (Besson and Chaouch, 1987; Kaplan and Driscoll, 1997). Although the ASH and FLP sensory neurons synapse with many of the same interneurons as the ALM and AVM mechanosensory neurons, nose touch employs a separate mode of synaptic transmission. ASH-mediated nose touch requires GLR-1, an AMPA-type glutamate receptor expressed by many postsynaptic targets of ASH and FLP including the AVA and AVD interneurons (Hart et al., 1995; Maricq et al., 1995). Mutations in *glr-1* block the response to nose touch, but do not alter avoidance to body touch or high osmolarity, even though these responses are also mediated by the AVA and AVD interneurons. We found that mutations in *unc-42* altered all of these behaviors. Nose touch and anterior body touch were severely diminished (Table 1), and osmotic avoidance was also reduced (data not shown).

ASH differentiation is altered in *unc-42* mutants

To determine if developmental defects in the mechanosensory neurons could account for these behavioral defects, we first examined the morphology and differentiation of mutant ALM,

AVM and ASH sensory neurons. UNC-86, a POU homeodomain protein required to specify ALM, AVM and FLP cell fate (reviewed by Ruvkun, 1997), was expressed normally in the mutants (data not shown). The axon trajectories of the *unc-42* and wild-type ALM and AVM neurons were also indistinguishable when animals were stained with an antiserum to α -tubulin (Piperno and Fuller, 1985).

The ASH neurons and several other sensory neurons with ciliated endings take up lipophilic fluorescent dyes such as DiI. When *unc-42* mutants were incubated in media containing DiI, the ASH neurons took up the dye normally, indicating that their ciliated ending were exposed. Although ASH axonal and dendritic morphology appeared normal in these animals (data not shown), we found that other markers for ASH differentiation were not expressed. The ASH neurons of *unc-42(e419)* mutants failed to express an *srb-6-gfp* transgene (Fig. 1), while the ADL

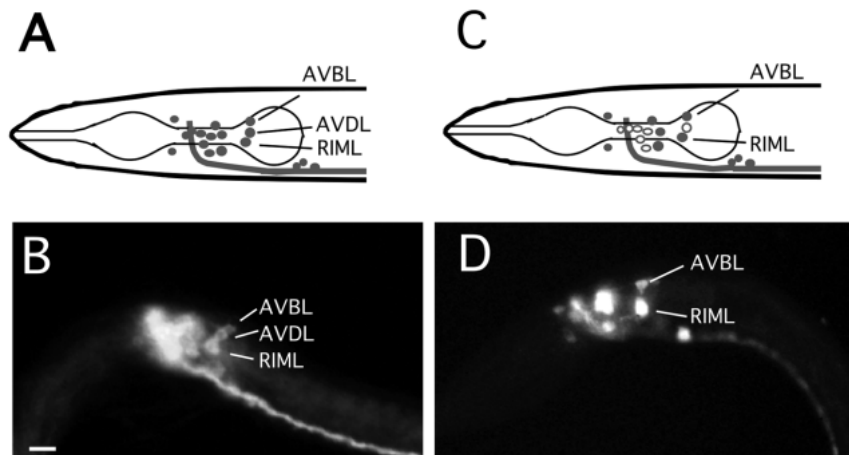


Fig. 2. *glr-1-gfp* transgene expression in *unc-42* mutant animals. The AVA, AVD and AVE interneurons and RMD and RME motor neurons of *unc-42* mutants fail to express *nuls1*, an integrated *glr-1-gfp* reporter transgene (Hart et al., 1995). (A,C) Diagram of the pattern of *glr-1-gfp* expression in the nematode head in (A) wild-type and (C) *unc-42* L1 larvae. 34 cells in the head express *glr-1-gfp* in wild-type worms (Hart and Kaplan, 1995; Maricq et al., 1995). Circles depict the position of cell bodies on the left side. Solid circles represent the cells that express *glr-1-gfp*; open circles represent cells that are present but fail to express the GFP reporter in *unc-42* mutants. (B,D) Fluorescence photomicrographs of left lateral views of (B) wild-type and (D) *unc-42(e419)* larvae showing expression of the *glr-1-gfp* transgene. Cells in the head that continue to express GLR-1 in the mutants are the URY, SMD, AVB, RIM, AVG, RIG and RIS neurons. Scale bar, 10 μ m.

and ADF sensory neurons expressed this transgene normally. ASH neurons of *unc-42* mutants also failed to express an *sra-6-gfp* transgene (Wightman et al., 1997; data not shown). *sra-6* and *srb-6* encode seven-transmembrane receptors that may function as chemoreceptors for the detection of volatile repellents (Troemel et al., 1995).

Differentiation of the AVA, AVD and AVE interneurons and the RME and RMD motor neurons is altered in *unc-42* mutants

Failures in ASH function could account for behavioral defects mediated solely by ASH, but cannot account for the defects in body touch or locomotion, or the severity of the nose-touch defects of *unc-42* mutants. If the ASH neurons of wild-type animals are killed by laser microsurgery, worms can still respond to nose touch 37% of the time (Kaplan and Horvitz, 1993). By contrast, *unc-42* mutants respond to nose-touch stimuli only 10% of the time (Table 1). These results predict that cells in addition to ASH contribute to the nose-touch defects of the mutants.

To determine if *unc-42* mutations also disrupted interneuron function, we tested whether *unc-42* mutants expressed a transcriptional *glr-1-gfp* reporter transgene. *glr-1* encodes an AMPA-type glutamate receptor expressed by all interneurons of the forward and backward locomotory circuit, as well as by a subset of head motor neurons (Hart et al., 1995; Maricq et al., 1995). We could not detect *glr-1-gfp* expression in the AVA, AVD and AVE interneurons in living mutants or in mutant worms that were fixed and stained with an anti-GFP antiserum to enhance GFP detection (Fig. 2). Identical results were obtained for *unc-42* mutants that express a functional GLR-1 receptor tagged with GFP (*nuls25*; Rongo et al., 1998). These interneurons receive synaptic input from ASH and FLP and provide output to motor neurons that control backward movement. The cell bodies of the interneurons were still present in the mutants, based on Nomarski microscopy of live animals and staining of fixed worms with DAPI (data not shown). However, we were unable to determine if the morphology of the interneuron axons along the ventral cord is normal in *unc-42* mutants because individual markers for these neurons are not available. The expression of the *glr-1-gfp* transgene was unaffected in AVB and PVC, interneurons that control forward motion (Fig. 2). Thus, the loss of GLR-1 receptor expression by the AVA, AVD and AVE interneurons contributes to the severe nose-touch phenotype of *unc-42* mutants.

Mutations in *unc-42* also disrupted *glr-1-gfp* expression in the six RMD and two RME motor neurons, which innervate head muscles (Fig. 2). The RME neurons are involved in foraging behavior, and the RMDs mediate a head withdrawal response to touch along the side of the nose

that is also dependent on *glr-1* (Hart et al., 1995). Because head mobility is defective in *unc-42* mutants, we did not test mutant animals for defects in head withdrawal.

unc-42 encodes a novel homeodomain protein

Altered expression of late differentiation genes in several *unc-42* mutant neurons suggested that *unc-42* may act to regulate gene expression. We cloned *unc-42* by transformation rescue (see Materials and Methods) and determined that it encodes a novel homeodomain protein of the paired-like class. The *unc-42* gene structure is depicted in Fig. 3A. Northern blot analysis of mixed stage *C. elegans* RNA using an RNA antisense probe generated from the *unc-42* cDNA revealed a single transcript of 1.2 kb (Fig. 3B). Sequence analysis of *unc-42* cDNAs predicted a protein product of 263 amino acids (Fig. 4A).

To confirm that this gene is *unc-42*, we sequenced four of the mutant *unc-42* alleles. The *e419* and *gm23* mutations are both GC to AT transitions that insert amber stop codons at position 10 and 48 of the homeodomain, respectively. The *e270* mutation converts a conserved valine to glutamic acid at position 47 of helix three (Fig. 4B, top). The weak allele *gm18* is a missense mutation in the region immediately C-terminal to the homeodomain (data not shown).

The alignment of the UNC-42 homeodomain with the homeodomains of related proteins is shown in Fig. 4B. The *C. elegans* homeoproteins most closely related to UNC-42 are CEH-10 and UNC-4 (Hawkins and McGhee, 1990; Miller et al., 1992). The homeodomain of UNC-42 is 68% identical to CEH-10 and 65% identical to UNC-4. UNC-42 exhibits the same degree of similarity to a number of homeoproteins of the paired-like classes from other species, including the vertebrate proteins, Cart-1, Phox2a and Arx (Zhao et al., 1993; Zellmer et al., 1995; Miura et al., 1997).

UNC-42 expression

To determine which cells express UNC-42 protein, we generated polyclonal antisera to UNC-42 in mice and rabbits to stain whole mounts of embryos, larvae and adult worms. We

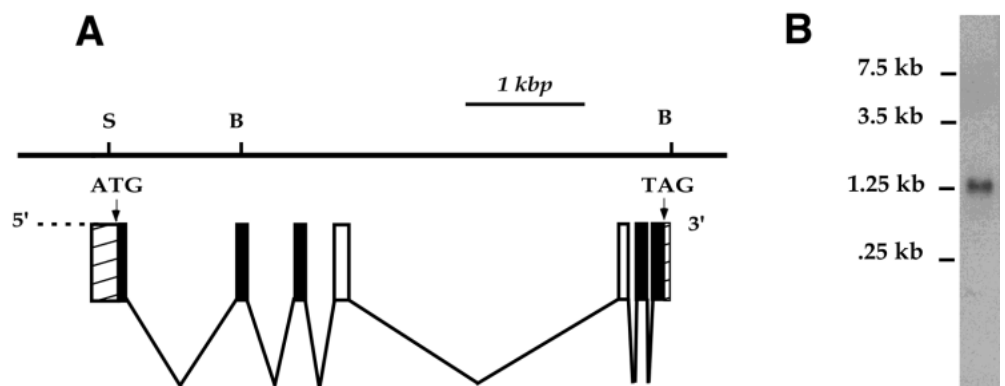
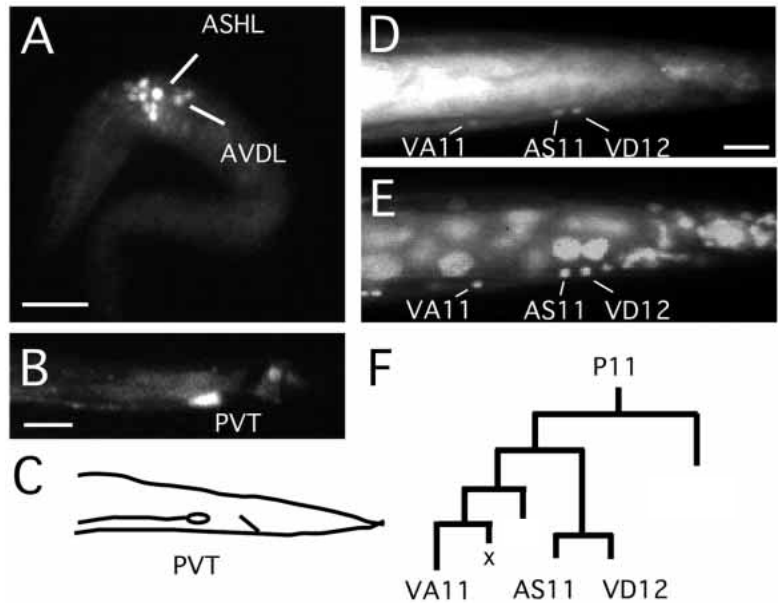


Fig. 3. *unc-42* gene structure. (A) Structure of the *unc-42* gene. At the top is a restriction map showing the positions of the *SalI* (S) and *BamHI* (B) sites. ATG and TAG are the predicted start and stop sites of translation, respectively. Boxes represent exons; V, introns; solid and open boxes, coding sequences; open boxes, homeodomain sequences; crosshatched boxes, 5' and 3' UTR sequences; dashed line, indication that we have not defined the precise 5' end of the mRNA. (B) northern hybridization analysis. A northern blot of poly(A)⁺ RNAs from a mixed stage culture was probed with an *unc-42* cDNA probe.

Fig. 5. *unc-42* is expressed in a subset of interneurons, sensory neurons and motor neurons in *C. elegans*. Cell identifications were made based on cell positions in (L1) larvae and double staining *glr-1-gfp* transgenic worms with a rabbit antiserum to GFP and mouse anti-serum to UNC-42. (A) Late stage (3½-fold) embryo stained with a mouse antiserum to UNC-42. The positions of the AVDL interneuron and the ASHL sensory neuron are indicated. Scale bar, 25 µm. (B) Expression of *unc-42-gfp* (*gmEx104*) transgene in the PVT interneuron of an L2 larva. PVT is a large, single interneuron located at the ventral midline of the pre-anal ganglion. (C) Diagram of the position of PVT in the tail. Scale bar, 10 µm. (D,E) UNC-42 is expressed in ventral cord motor neurons derived from P11. Wild-type adult hermaphrodites were double-stained with (D) rabbit anti-UNC-42 antisera to detect cells expressing UNC-42, and (E) DAPI to visualize the nuclei of all cells. Scale bar, 25 µm. (F) Lineage of the P11 blast cell. UNC-42 is not expressed in postembryonic motor neurons that are generated from P2-10 or P12, but is expressed by all three motor neurons derived from P11.



P2-P10 or P12. In the tail, *unc-42-gfp* (*gmEx104*) is strongly expressed in PVT (Fig. 5B).

***unc-42* functions in AB.a for response to light body touch and in AB.p for locomotion**

UNC-42 was expressed by AVA and AVD interneurons, which transmit stimuli from the ALM and AVM mechanosensory neurons and the ASH and FLP sensory neurons to motor neurons. To determine whether defects in the interneurons are responsible both for the sensory and locomotory defects of *unc-42* mutants, we analyzed the behavior of animals that were mosaic for *unc-42* function (Materials and Methods).

We were surprised to find that the Unc and Mec phenotypes were separable in the mosaic animals, reflecting distinct sites of *unc-42* function. We could isolate mosaic animals that were Unc but not Mec, as well as animals that were Mec but not Unc (Fig. 6). AVA and AVD, the interneurons that mediate backward motion, are derived from the AB.a blastomere. The AVB and PVC interneurons that control forward movement, ventral cord motor neurons that innervate body wall muscles, and many of the neurons that regulate head movement are all descendants of the AB.p blastomere (Sulston et al., 1983). 11/11 mosaic worms that failed to respond to light touch to the anterior body, but moved normally, had lost wild-type *unc-42* gene activity in AB.a (Fig. 6A). Six of these losses were in AB.aala, producing mosaic animals that had mutant AVD interneurons, wild-type AVM and ALM mechanosensory neurons and wild-type motor neurons. By contrast, 12/12 mosaic worms that were Unc, but responded to light touch, had lost wild-type *unc-42* function in the AB.p lineage (Fig. 6B). A severe uncoordinated phenotype was observed only when all cells derived from AB.p were mutant for *unc-42*. Animals with single losses of wild-type gene function in AB.p descendants were wild type, and the animals with multiple losses in AB.p descendants exhibited only weak defects in locomotion.

These results are consistent with the results of laser killing experiments, which showed that most members of a single motor neuron class or multiple classes must be killed to

generate a severe uncoordinated phenotype (Chalfie et al., 1985). Because motor neurons are generated at multiple points in the AB.p cell lineage (Fig. 6B), only early losses in the lineage would affect gene function in a significant number of ventral cord motor neurons. In addition, some of the motor neurons that innervate head muscles and interneurons that coordinate head movement also express UNC-42 and are derived from AB.p.

Genetic mosaic analysis supports the hypothesis that *unc-42* acts cell-autonomously in the AVD interneurons for the response to body touch and is likely to act cell-autonomously in motor neurons for locomotion. However, we did not detect overt differentiation defects in ventral cord motor neurons. We examined the VD and DD motor neurons of *unc-42* mutants for expression of the neurotransmitter GABA, but did not detect any significant abnormalities in the placement of ventral cord axons or commissures (data not shown). Expression of CHA-1 and UNC-17, markers for cholinergic motor neuron differentiation, were also unaffected in *unc-42* animals (J. Duerr and J. Rand, personal communication).

***unc-42(e270)* and *unc-42(gm23)* mutations alter gene activity**

We showed previously that *unc-42* is required for correct formation of the left ventral nerve cord of *C. elegans* (Wightman et al., 1997). The *C. elegans* nerve cord is asymmetric, with only four axons in the left bundle and more than 40 axons extending along the right bundle (White et al., 1985). Severe *unc-42* alleles exhibited defects in pathfinding by three of the four neurons that contribute axons to the left nerve cord bundle: the HSNL motor neuron, and the PVQL and AVKR interneurons. We also found that in 20-30% of *e270* and *gm23* hermaphrodites, but not in other *unc-42* mutants, PVPR failed to pioneer the left nerve tract. The PVPR pioneering defect of *gm23* and *e270* hermaphrodites results in a more severe HSNL defect: 80-86% of the HSNL axons from *gm23* and *e270* hermaphrodites display pathfinding errors, whereas only 60-65% of the HSNL axons of other strong *unc-42*

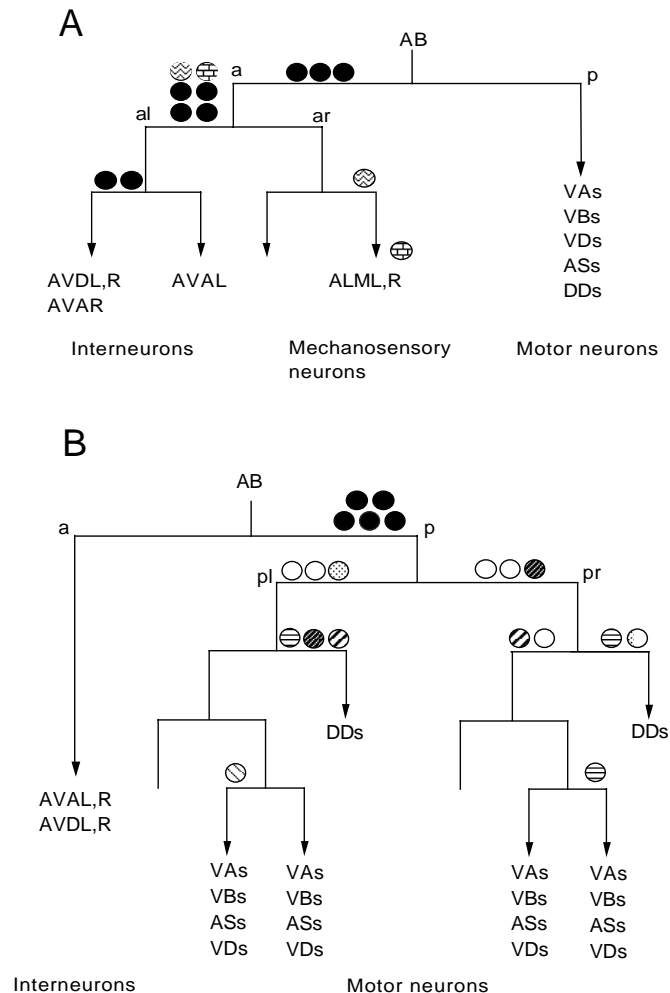


Fig. 6. Genetic mosaic analysis of *Unc-42* *Unc* and *Mec* phenotypes. *unc-42* function is required in interneurons that mediate response to gentle anterior touch and in motor neurons that control locomotion. Mosaic analysis was performed as described in Materials and Methods. (A) Solid black circles, single losses of the *ctDp11* duplication in the cell lineage that resulted in the anterior *Mec* (mechanosensory defective) phenotype; patterned symbols, multiple losses in the cell lineage that resulted in a *Mec* phenotype; circles with the same patterns, multiple losses in the same mosaic animal. In 11/11 *Mec* non-*Unc* mosaic animals, wild-type *unc-42* function was lost in the AB.a lineage. Single losses of *ctDp11* at AB.aala were sufficient to cause *Mec* defects. (B) Solid black circles, single losses of the *ctDp11* duplication in the cell lineage that resulted in an *Unc* (uncoordinated locomotion) phenotype; open circles, single losses of *ctDp11* in the cell lineage that resulted in wild-type movement; patterned circles, multiple losses in the cell lineage observed in weakly uncoordinated animals; circles with the same patterns, multiple losses in the same mosaic animal. The *Unc*, non-*Mec* phenotype was observed only in mosaic animals that lost the duplication in AB.p. Only single losses of *ctDp11* very early in the AB.p lineage were sufficient to generate a severe *Unc* phenotype.

hermaphrodites, such as *e419*, pathfind abnormally (Wightman et al., 1997).

In light of these findings, the prediction from molecular analysis that *e419* is the best candidate for a null allele came as a surprise. The *e419* allele is a nonsense mutation at the

beginning of the homeodomain, whereas the *e270* and *gm23* alleles are missense and nonsense mutations, respectively, in the third helix of the homeodomain. These observations raised the possibility that the *gm23* and *e270* mutations not only eliminate, but also alter *unc-42* function. In this model, altered *unc-42* function results in aberrant PVPR pioneering. To test this hypothesis, we examined HSNL axon pathfinding in *unc-42* heterozygous hermaphrodites. In 10% of *e270/+* and 12% of *gm23/+* worms, the HSNL axon crossed the midline and extended along the right ventral nerve cord ($n=50$). By comparison, less than 2% of the *unc-42(e419)/+* worms or wild-type controls showed this defect in HSNL pathfinding ($n=50$). HSNL pathfinding was also normal in animals hemizygous for *sDf29*, a deficiency chromosome that deletes *unc-42* ($n=25$).

DISCUSSION

UNC-42 belongs to a family of homeodomain proteins that regulate neuronal differentiation

unc-42 encodes a novel homeodomain protein of the paired-like class. A growing body of evidence suggests that homeoproteins of this class play essential roles in defining neuron subtype identity. Other members of this family include UNC-4, CEH-10 and UNC-30 in *C. elegans*, and Phox2a in vertebrates. UNC-4 controls the differentiation of the VA motor neurons of *C. elegans* by specifying synaptic input (White et al., 1992). In *unc-4* mutants, the VAs receive input from the interneurons that normally synapse onto their sister cells, the VBs, but retain the wild-type VA pattern of axon projections and output to motor neurons. CEH-10 regulates migration and differentiation of the bipolar canal-associated neurons (CANs) and is closely related to Chx10 in mouse, a paired-like protein required for differentiation of bipolar neurons in the vertebrate retina (Forrester et al., 1998; Liu et al., 1994; Burmeister et al., 1996). The *C. elegans* gene *unc-30* regulates differentiation of type-D motor neurons. In *unc-30* mutants, type-D axonal morphology is altered, and the type-D motor neurons fail to make appropriate synaptic connections (J. White, personal communication) and to express their neurotransmitter GABA (McIntire et al., 1993a,b; Jin et al., 1994). Similarly, mice mutant for the paired-like homeodomain gene *Phox2a* exhibit defects in catecholamine biosynthesis and fail to express the Ret receptor for GDNF in specific tissues (Morin et al., 1997).

Several of these homeoproteins appear to act as direct transcriptional regulators of late neuronal differentiation genes. Arix, the rat homolog of Phox2a has been shown to activate in vitro transcription from the promoter of the dopamine beta-hydroxylase and tyrosine hydroxylase genes, which encode dopamine biosynthetic enzymes (Zellmer et al., 1995). In *C. elegans*, UNC-30 may be a direct regulator of GAD and other genes required for GABAergic differentiation (C. Eastman and Y. Jin, personal communication).

unc-42 represents a new neuronal identity gene in *C. elegans*. UNC-42 protein can be detected in neuronal precursor cells at 260 minutes and by comma stage is expressed at high levels in a subset of the *C. elegans* embryonic nervous system. Although *unc-42* is expressed by about 15% of *C. elegans* neurons, loss of *unc-42* function in a few key neurons of the

mechanosensory circuit is responsible for many of the behavioral defects displayed by the mutants.

unc-42 is required for ASH cell fate

We found that UNC-42 is expressed at high levels in the ASH sensory neurons and is required for ASH development. *unc-42* mutants are defective for multiple ASH-mediated behaviors and fail to express the putative chemoreceptor genes *sra-6* and *srb-6* (Troemel et al., 1995). It is unlikely, however, that loss of *unc-42* function in the ASH neurons is sufficient to cause the severe defects in nose-touch avoidance seen in the mutants. 37% of wild-type animals still respond to nose touch when the ASH neurons are killed, whereas *unc-42* mutants exhibit almost no response to nose touch, a phenotype seen in animals lacking both ASH and FLP (Kaplan and Horvitz, 1993). Because UNC-42 is expressed by ASH and the AVA and AVD interneurons, and not by FLP, the inability of the mutants to respond to nose touch is probably due to defects both in the ASH sensory neurons and the interneurons.

unc-42 specifies AVA, AVD and AVE cell fate

Distinct neural circuits regulate backward and forward movement in *C. elegans* (Chalfie et al., 1985). AVA and AVD interneurons transmit stimuli from anterior sensory neurons to motor neurons that control backward locomotion, and AVB and PVC interneurons transmit stimuli from posterior sensory neurons to motor neurons that elicit forward motion. Our results show that separate genetic programs control the development of the circuits for forward and backward motion, with *unc-42* controlling the circuit for backward movement by regulating AVA, AVD and AVE interneuron differentiation.

The axons of the AVA, AVD and AVE interneurons extend around the nerve ring and along the right ventral nerve cord. They are exclusively postsynaptic in the nerve ring and receive input from diverse classes of mechanosensory and chemosensory neurons in the nose and head of *C. elegans* (White et al., 1986). For instance, the AVDs are synaptic targets of the ASH, FLP and ADL sensory neurons and form gap junctions with the AVM and ALM touch cells. Our results demonstrate that *unc-42* plays an essential role in AVA, AVD and AVE differentiation. First, *unc-42* mutations cause severe defects in behaviors that are mediated by AVA and AVD, such as nose touch and touch along the anterior body, but do not affect behaviors dependent on AVB and PVC. Second, genetic mosaic analysis indicates that *unc-42* function for anterior body touch is required in the AB.a blastomere, which generates both AVD neurons and one of the AVA neurons. Third, *unc-42* is expressed by the AVA, AVD and AVE interneurons, but not by the AVB and PVC interneurons. Finally, *unc-42* mutations abolish expression of the GLR-1 glutamate receptor by the AVA, AVD and AVE neurons, but do not alter GLR-1 expression by AVB and PVC. Thus, *unc-42* is required only for the function of the backward command interneurons.

unc-42 regulates the GLR-1 mediated neural circuitry for nose touch

The *C. elegans* GLR-1 glutamate receptor is related to mammalian AMPA class (GluR) receptor subunits and is expressed in about 40 interneurons and motor neurons (Hart et al., 1995; Maricq et al., 1995). Glutamate receptors play critical roles in nervous system function, but transcription

factors that regulate glutamate receptor expression have not been identified. The defects in GLR-1 expression observed in *unc-42* mutants raise the possibility that *unc-42* is a direct regulator of glutamate receptors in *C. elegans*.

unc-42 mutations eliminated *glr-1-gfp* transgene expression in interneurons and motor neurons that are part of the circuitry for nose touch: the RMD motor neurons and the AVA, AVD and AVE interneurons. These neurons mediate two distinct avoidance behaviors to nose touch that are dependent on GLR-1: backward body movement and head withdrawal (reviewed by Kaplan and Driscoll, 1997). Backward movement is mediated through the AVA and AVD interneurons, which form gap junctions with body wall motor neurons. Head withdrawal requires the RMD motor neurons, which form gap junctions with the AVEs and receive input directly from the sensory neurons IL1, OLL and OLQ (White et al., 1986).

It is interesting that *unc-42* mutations disrupt GLR-1 expression by neurons that are part of the neural circuitry for nose touch, but do not affect GLR-1 expression by other neurons. Similar results were observed for the control of GABA synthesis by *unc-30*. Although ectopic expression of *unc-30* can activate synthesis of the neurotransmitter GABA both in neurons and epithelial cells, *unc-30* is not a global regulator of GABAergic differentiation in *C. elegans* (Jin et al., 1994). *unc-30* is required for GABA biosynthesis in the DD and VD motor neurons that are part of the body wall circuitry, but does not control other GABAergic neural circuits in the head and tail of *C. elegans*. In a similar fashion, *unc-42* does not act as a global regulator of GLR-1, but is required for GLR-1 expression only in neural circuitry for nose-touch responses.

unc-42 regulates multiple neural circuits for locomotion

unc-42 mutants have complex movement defects that predict *unc-42* function is required in more than one locomotory circuit. Both backward and forward movement are abnormal, and coordination of anterior body movement is particularly affected. Mutants cannot propagate a normal sinusoidal wave through the head, have difficulty backing up and often kink ventrally. Several observations argue that these behavioral defects are due to loss of *unc-42* function in multiple classes of motor neurons, and possibly also to loss of function in interneurons that coordinate head and body wall movement. First, the uncoordinated phenotype of *unc-42* mutants is more complex than the defects observed when any single class of ventral cord motor neuron is removed by laser microsurgery (Chalfie et al., 1985). Second, genetic mosaic analysis shows that *unc-42* function must be lost at multiple positions in the AB.p lineage to generate a severely uncoordinated animal. Finally, *unc-42* has a complex expression pattern, including the embryonic DD motor neurons, motor neurons derived from P11 and three of the four classes of motor neurons in the head that innervate both head muscles and anterior body wall muscles: the RMD, RME and SMB motor neurons. *unc-42* is also expressed by the SIB and SAA interneurons that may function in the coordination of head and body wall muscles.

gm23 and e270 alter unc-42 gene function

Mutations in *unc-42* disrupt axon pathfinding in the left ventral nerve cord bundle of *C. elegans* (Wightman et al., 1997). We did not detect UNC-42 protein in the PVP, PVQ or HSN

neurons in larvae or adults, suggesting that UNC-42 expression by these neurons may be transient or below detection with our reagents, or that the pathfinding defects of these neurons is non-autonomous. Our genetic analysis suggests that altered *unc-42* activity in the *e270* and *gm23* mutants leads to PVPR axon pioneering defects. During embryogenesis, the PVPR neuron first extends its axon contralaterally across the midline and then anteriorly along the ventral epithelial surface to pioneer the left ventral nerve cord bundle (Durbin et al., 1987). This step is dependent on PVT (Wadsworth et al., 1996), an interneuron at the ventral midline that expresses *unc-42-gfp*. The additional pathfinding defects that we observe in *gm23* and *e270* mutants could occur if altered *unc-42* activity affected PVT differentiation, disrupting interactions between the PVPR growth cone and PVT. Because UNC-42 protein is also expressed at low levels in epithelial cells that provide a substratum for the PVPR axon, another possibility is that epithelial defects disrupt PVPR axon guidance in *gm23* and *e270* mutants.

The *e270* and *gm23* mutations are in adjacent, highly conserved positions of the UNC-42 recognition helix. The *e270* mutation converts valine 47 of helix 3 to glutamic acid, and *gm23* replaces tryptophan 48 with a stop codon, eliminating the C-terminal half of the protein, including most of helix three and a putative activation domain. Residues 47-49 and 51 are invariant in all paired and paired-like homeodomains. Analysis of other homeoproteins has shown that residues at position 47 and 51 interact with the major groove of the DNA and are required for high-affinity binding (Gehring et al., 1994). Because the *e270* and *gm23* mutations are likely to greatly reduce DNA-binding affinity or specificity, these two alleles probably eliminate normal *unc-42* function. This hypothesis is supported by the behavioral phenotypes of *unc-42* homozygotes: the Unc and Mec defects are similar in *gm23*, *e270* and *e419* mutants. In addition, expression of a transcriptional *unc-42-gfp* fusion gene was abolished in adults in all three mutant strains (data not shown), suggesting that *unc-42* regulates its own expression and that none of the mutant proteins is able to perform this function. However, low levels of UNC-42 protein can still be detected in some *gm23* and *e270* mutant animals stained with UNC-42 antisera (data not shown).

Two hypotheses can account for altered function produced by the *gm23* and *e270* lesions. Sequences N-terminal to the homeodomain and in helices 1 and 2 would be normal in both mutant proteins. These sequences have been shown to contribute to dimerization and DNA binding in other homeoproteins (see Gehring et al., 1994 for review). One possibility is the aberrant *gm23* and *e270* proteins sequester cofactors that are necessary for other processes. Alternatively, the binding specificity of the mutant proteins could be altered, causing them to bind to different DNA sequences and resulting in phenotypes distinct from those produced by loss of *unc-42* function.

Dominant mutations have also been identified in other paired-like homeoproteins. An autosomal dominant form of cone-rod dystrophy (CORDII) in humans is caused by mutations in *CRX* (Freund et al., 1997; Swain et al., 1997). However, it is not known if the photoreceptor degeneration caused by these mutations is due to haploinsufficiency or altered gene activity.

The role of UNC-42 in neuronal differentiation

How does *unc-42* act to specify neuronal fate? In *unc-42* mutants, affected neurons were present and did not appear to adopt other neuronal fates. Affected neurons, however, fail to differentiate fully. The ASH phenotypes of *unc-42* mutants are particularly informative. The mutant ASH neurons were grossly normal in morphology, extending a dendrite anteriorly to the nose and an axon to the nerve ring, the normal target for this class of sensory neuron. The mutant neurons were also able to take up DiI, indicating that their sensory endings were exposed to the environment. The cells, however, failed to express the putative chemosensory receptor SRB-6. Thus, *unc-42* is required for the expression of some ASH differentiation traits, but it is not essential for determining ASH identity as a sensory neuron. Several models could account for our results. *unc-42* may function primarily at a specific time late in neuronal differentiation. Alternatively, *unc-42* may be required throughout neuronal development, and regulate specific traits in parallel with factors that determine more general features of neuron identity.

We are grateful to Leilani Miller and Barbara Meyer for the cDNA library, Andy Fire and Cori Bargmann for plasmids, Gary Ruvkun and Michael Finney for UNC-86 antiserum, Josh Kaplan for *glr-1-gfp* transgenic strains, and Nancy Hawkins for the *srb-6-gfp* transgenic strain. We also thank Yishi Jin and Jim Rand for sharing their unpublished observations, and Piali Sengupta and Josh Kaplan for comments on the manuscript. Some strains were provided by the *Caenorhabditis* Genetics Center, St. Paul, MN. This work was supported by National Institute of Health grant NS32057 to G. G.

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