Developmental Cell, Vol. 5, 621–633, October, 2003, Copyright ©2003 by Cell Press

Otx/otd Homeobox Genes Specify Distinct Sensory Neuron Identities in *C. elegans*

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Summary

The mechanisms by which the diverse functional identities of neurons are generated are poorly understood. *C. elegans* responds to thermal and chemical stimuli using 12 types of sensory neurons. The *Otx/otd* homolog *ttx-1* specifies the identities of the AFD thermosensory neurons. We show here that *ceh-36* and *ceh-37*, the remaining two *Otx*-like genes in the *C. elegans* genome, specify the identities of AWC, ASE, and AWB chemosensory neurons, defining a role for this gene family in sensory neuron specification. All *C. elegans* *Otx* genes and rat *Otx1* can substitute for *ceh-37* and *ceh-36*, but only *ceh-37* functionally substitutes for *ttx-1*. Functional substitution in the AWB neurons is mediated by activation of the same downstream target *lim-4* by different *Otx* genes. Misexpression experiments indicate that although the specific identity adopted upon expression of an *Otx* gene may be constrained by the cellular context, individual *Otx* genes preferentially promote distinct neuronal identities.

Introduction

Animals must recognize and respond to multiple, complex sensory stimuli in order to survive and reproduce. Environmental signals are first recognized by peripheral sensory neurons, which are remarkable in their functional diversity. For example, components of complex olfactory stimuli are detected through the distinct chemosensory functions of olfactory neurons, thereby enabling animals to distinguish and discriminate among thousands of odorants (Sicard and Holley, 1984; Krautwurst et al., 1998; Malnic et al., 1999). The developmental mechanisms by which this functional complexity is achieved are not fully understood.

The nematode *C. elegans* exhibits sensory responses to mechanical, thermal, and chemical stimuli (Bargmann and Mori, 1997; Driscoll and Kaplan, 1997). Multiple chemicals of diverse structures are recognized by 11 pairs of sensory neurons in the bilateral amphid sensory organs of the head. These include the AWA, AWB, and AWC neurons, which respond to different sets of volatile odorants, and the ASE neurons, which sense water-soluble chemicals (Bargmann and Horvitz, 1991; Bargmann et al., 1993; Troemel et al., 1997; Chou et al., 2001). A twelfth pair of amphid neurons, the AFD neurons, sense temperature (Mori and Ohshima, 1995). Each of these neuron types is distinguished by the expression of cell-specific signaling and structural genes, which specify their distinctive sensory functions as well as their unique morphologies and connectivities (Ward et al., 1975; Perkins et al., 1986; White et al., 1986; Bargmann and Mori, 1997; Troemel, 1999). Members of transcription factor families, including the LIM homeodomain and nuclear receptor proteins, specify the subtype identities of a set of sensory neurons (Sengupta et al., 1994; Baran et al., 1999; Sagasti et al., 1999; Sarafi-Reinach et al., 2001; Satterlee et al., 2001; Uchida et al., 2003). However, the developmental pathways in which these genes function are relatively uncharacterized. Moreover, genes required for specification of identity of additional sensory neuron types remain to be identified.

Members of the well-conserved OTD/OTX family of homeodomain proteins have been implicated in the development and patterning of the central nervous system, including sensory structures, in multiple species (Simeone, 1998; Hirth and Reichert, 1999; Acampora et al., 2000; Simeone et al., 2002). Gene replacement experiments have suggested that the *Otx1* and *Otx2* genes in mice can largely functionally substitute for each other, even though $Otx1^{-/-}$ and $Otx2^{-/-}$ animals exhibit markedly distinct phenotypes, indicating that their distinct mutant phenotypes are primarily a consequence of divergence in expression patterns as opposed to protein functions (Acampora et al., 1998b, 1999, 2000). Intriguingly, despite the evident dissimilarites in the structures of the mammalian and *Drosophila* brains, the *Drosophila otd* gene can also substitute for both *Otx1* and *Otx2* functions in mice and vice versa (Acampora et al., 1998a, 2001; Leuzinger et al., 1998; Nagao et al., 1998). It is unclear whether this functional equivalence arises from the activation of identical downstream target genes.

The *C. elegans* genome is predicted to encode three *Otx*-related genes. We previously showed that the *Otx* gene *ttx-1* is both necessary and sufficient for the specification of identity of the AFD thermosensory neurons (Satterlee et al., 2001). Here, we show that the remaining two *Otx* genes *ceh-36* and *ceh-37* specify the identities of the AWC and AWB olfactory neurons, respectively. *ceh-36* also plays a role in the functional diversification of the left and right ASE chemosensory neurons. We demonstrate that all three *Otx* genes in *C. elegans* as well as rat *Otx1* can functionally substitute for each other in the specification of AWC and AWB but not AFD identity; in the AWB neurons, this functional substitution arises as a consequence of activation of the same downstream gene. Finally, our results suggest that the ability of an *Otx*-like gene to promote a specific cellular identity

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in *C. elegans* is dictated both by the cellular context in which it is expressed and by intrinsic differences in protein function.

Results

Isolation of *ceh-36* and *ceh-37* Alleles

The *oy45* allele was isolated in a screen for mutants with altered expression of the AWB-specific marker *str-1::gfp*, whereas the *ky640* and *ky646* alleles were isolated from screens for mutants with altered expression of the AWC marker *str-2::gfp* (Figures 1A and 1B). Genetic mapping, genomic rescue, and sequencing of mutant strains demonstrated that *oy45* represents an allele of *ceh-37*, and *ky640* and *ky646* are alleles of *ceh-36* (see Experimental Procedures). *ceh-36* and *ceh-37* are predicted to encode members of the OTX/OTD family of homeodomain proteins. Subsequent to this work, two additional *ceh-37* alleles (*ok272* and *ok642*) were isolated by the *C. elegans* Gene Knockout Consortium. *ceh-37(oy45)*, *ceh-37(ok642)*, and *ceh-36(ky646)* likely represent null alleles (Figure 2A).

The homeodomains (HDs) of CEH-36 and CEH-37 share 59% identity and 72% similarity and contain the lysine characteristic of this family at position 9 of the third helix (Figure 2B) (Galliot et al., 1999). However, the CEH-36 HD contains an atypical three amino acid insertion (VIT) in the predicted third helix (Figure 2B). Sequences other than the HD share significantly lower homology with each other or with OTX proteins from other species. Phylogenetic analysis suggests that CEH-36 and CEH-37 are more diverged from other OTX-related proteins than TTX-1, the third OTD/OTX member in *C. elegans* (Satterlee et al., 2001) (see Supplemental Figure S1 in the Supplemental Data available at http://www.developmentalcell.com/cgi/content/full/5/4/621/DC1).

Mutations in *ceh-37* Affect AWB-Specific Gene Expression and Olfactory Functions

ceh-37 mutants exhibited defects in multiple aspects of AWB olfactory neuron specification. All three *ceh-37* mutants exhibited strongly reduced expression of the AWB-specific *str-1::gfp* transgene (Table 1). In addition, expression of the *odr-1* receptor guanylyl cyclase, which is normally required for the sensory functions of the AWB and AWC neurons (L'Etoile and Bargmann, 2000), was lost specifically in the AWB neurons in multiple *ceh-37* alleles (Table 1). Reduction of expression of both *str-1::gfp* and *odr-1::dsRed* was evident as soon as marker expression could be scored in the first larval stages. The LIM homeobox gene *lim-4* has been previously shown to regulate AWB olfactory neuron identity, and a *lim-4::gfp* fusion gene is expressed in the AWB and additional neuron types (Sagasti et al., 1999). Expression of the *lim-4::gfp* transgene was abolished in the AWB neurons to the same extent as *str-1::gfp* in *ceh-37* alleles (Table 1), indicating that CEH-37 acts upstream of *lim-4* to specify AWB identity. Consistent with this model, expression of *lim-4* in the AWB neurons in *ceh-37* mutants was sufficient to restore *str-1* expression (data not shown). The loss of AWB-specific gene expression was incompletely penetrant in all three mutant strains, suggesting that CEH-37 may act together with an unknown gene(s) to regulate AWB identity.

The AWB neurons mediate avoidance responses to the volatile repellent 2-nonanone (Troemel et al., 1997). To investigate whether loss of AWB-specific gene expression correlated with a failure to avoid 2-nonanone, we examined the responses of individual *ceh-37(oy45)* mutant animals in which the expression of *str-1* was either maintained or lost in both AWB neurons. *ceh-37* mutants retaining *str-1* expression in both AWB neurons exhibited significantly greater avoidance of 2-nonanone than animals lacking *str-1* expression in both AWB neurons, indicating a correlation between loss of gene expression and neuronal function (Figure 1C). *ceh-37* mutants responded normally to chemicals sensed by other chemosensory neurons (data not shown).

The expression of markers for the AWA, AWC, ASH, ASI, and ASE sensory neuron types was unaffected in *ceh-37* mutants (Table 1 and data not shown). However, mutations in *ceh-37* weakly affected the expression of a gene in the AFD thermosensory neurons and significantly affected the expression of a gene in the ADF sensory neurons (Table 1). Loss of marker expression in the AWB and their ADF lineal siblings was not correlated (data not shown), suggesting that CEH-37 likely affects the differentiation of these two sister neuron types independently.

The AWB Neurons Retain General Sensory Neuronal Characteristics but Do Not Adopt a Specific Alternate Fate in *ceh-37* Mutants

In the absence of LIM-4 function, the AWB neurons adopt characteristics of the AWC olfactory neurons (Sagasti et al., 1999). Since *ceh-37* acts upstream of *lim-4* to regulate AWB neuronal identity, we asked whether the AWB neurons also adopted an AWC-like fate in *ceh-37* mutants. Unlike the AWB neurons in *lim-4* mutants, the AWB neurons in *ceh-37* mutants failed to misexpress the AWC marker *str-2* (Table 1). Moreover, the loss of *odr-1* expression in the AWB neurons is inconsistent with these neurons either retaining the AWB identity or adopting an AWC identity (Table 1). In addition, in *lim-4* mutants, adoption of AWC identity by the AWB neurons is associated with a loss in dye-filling ability (Sagasti et al., 1999; Perkins et al., 1986). However, the majority of affected AWB neurons in *ceh-37* mutants retained the ability to dye fill (75% of AWB neurons that fail to express *str-1::gfp* dye fill; n = 24). By these criteria, the affected AWB neurons in *ceh-37* mutants do not adopt an AWC-like identity.

lim-4 acts prior to L1 stages to repress *str-2* expression in the AWB neurons (Sagasti et al., 1999). Transient early *lim-4* expression in the affected AWB neurons in *ceh-37* mutants may be sufficient to repress *str-2* expression. Alternatively, in the absence of LIM-4 function, CEH-37 may promote the AWC fate in the AWB neurons. To distinguish between these possibilities, we examined the expression of *str-2* in *lim-4 ceh-37* double mutants. In the first model, the *str-2* misexpression phenotype of *lim-4 ceh-37* double mutants is expected to be similar to that of *lim-4* mutants alone. The second model predicts that misexpression of *str-2* would be observed

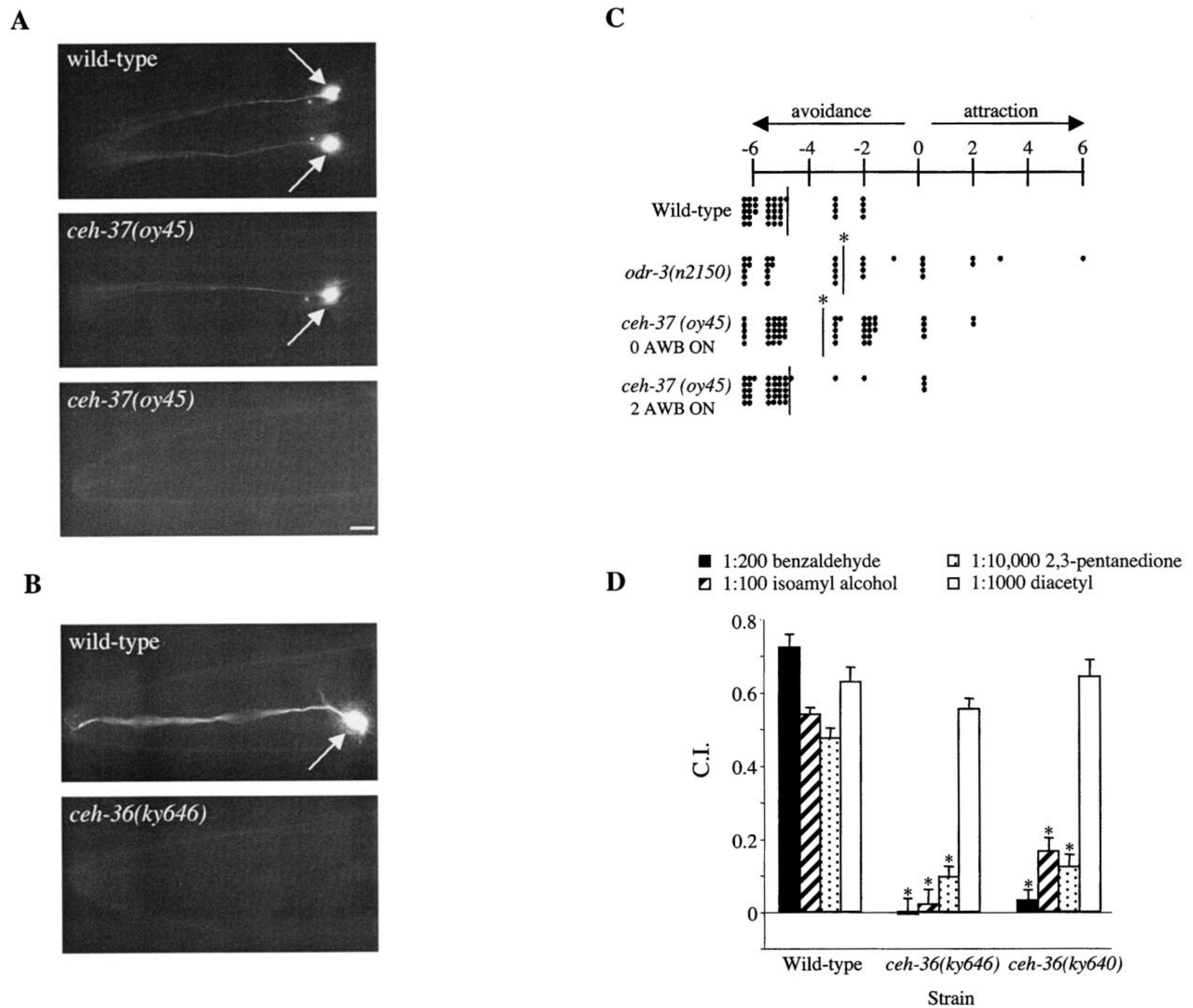

Figure 1. *ceh-37* and *ceh-36* Mutants Exhibit Defects in AWB- and AWC-Specific Marker Expression and Olfactory Functions

(A) Expression of a stably integrated *str-1::gfp* marker in both AWB neurons in wild-type (top) and in one or neither AWB neuron in *ceh-37(oy45)* mutants (middle and bottom). Top-down view; anterior is at left. Arrows indicate neuronal cell bodies. Scale bar, 10 μm.

(B) Expression of a stably integrated *str-2::gfp* marker in one AWC olfactory neuron in wild-type (top) and *ceh-36(ky646)* mutants (bottom). Lateral view; anterior is at left.

(C) Each circle represents the response of a single animal to a point source of 1 μl of a 1:10 dilution of 2-nonanone. Animals were assigned a score between −6 and +6, where −6 represents the strongest avoidance response. The vertical line represents the mean response. For *ceh-37(oy45)* mutants, the responses of animals expressing *str-1::gfp* in both (2 AWB ON) or no (0 AWB ON) AWB neurons were examined. *Odr-3* encodes a G protein subunit required for AWB-mediated olfactory responses (Troemel et al., 1997). *Responses that are different from those of wild-type and *ceh-37(oy45)* (2 AWB ON) animals at $p < 0.005$. All strains except *odr-3(n2150)* contain stably integrated copies of *str-1::gfp*.

(D) Responses of animals to a point source of the indicated odorants. Each data point is the mean of at least four independent assays using ∼100 animals each. Error equals the SEM. *Responses that are different from wild-type at $p < 0.001$. C.I., chemotaxis index. All strains contain stably integrated copies of *str-2::gfp*.

only in those AWB neurons that are affected by the loss of *lim-4* but unaffected by the loss of *ceh-37* due to its partial penetrance. As shown in Supplemental Table S1, the number of AWB neurons misexpressing *str-2* in the double mutants was similar to the number of AWB neurons which retained *str-1* expression in *ceh-37* mutants. These results suggest that CEH-37 acts to confer an AWC-like fate in the AWB neurons upon loss of *lim-4* expression.

The AWB neurons in *ceh-37* mutants also did not express markers specific for the AWA, AWC, AFD, ASH, ADF, ASE, or ASI neurons, since markers for these neuron types were not ectopically expressed in *ceh-37* mutants (Table 1 and data not shown). Moreover, when visualized by dye filling, the neurons retained their bipolar sensory neuronal morphology, indicating that the AWB neurons also did not adopt the fates of nonsensory or nonneuronal cells in *ceh-37* mutants. These results suggest that, in the absence of *ceh-37* function, the AWB neurons may retain "generic" sensory neuronal properties but fail to express a specific sensory neuron identity.

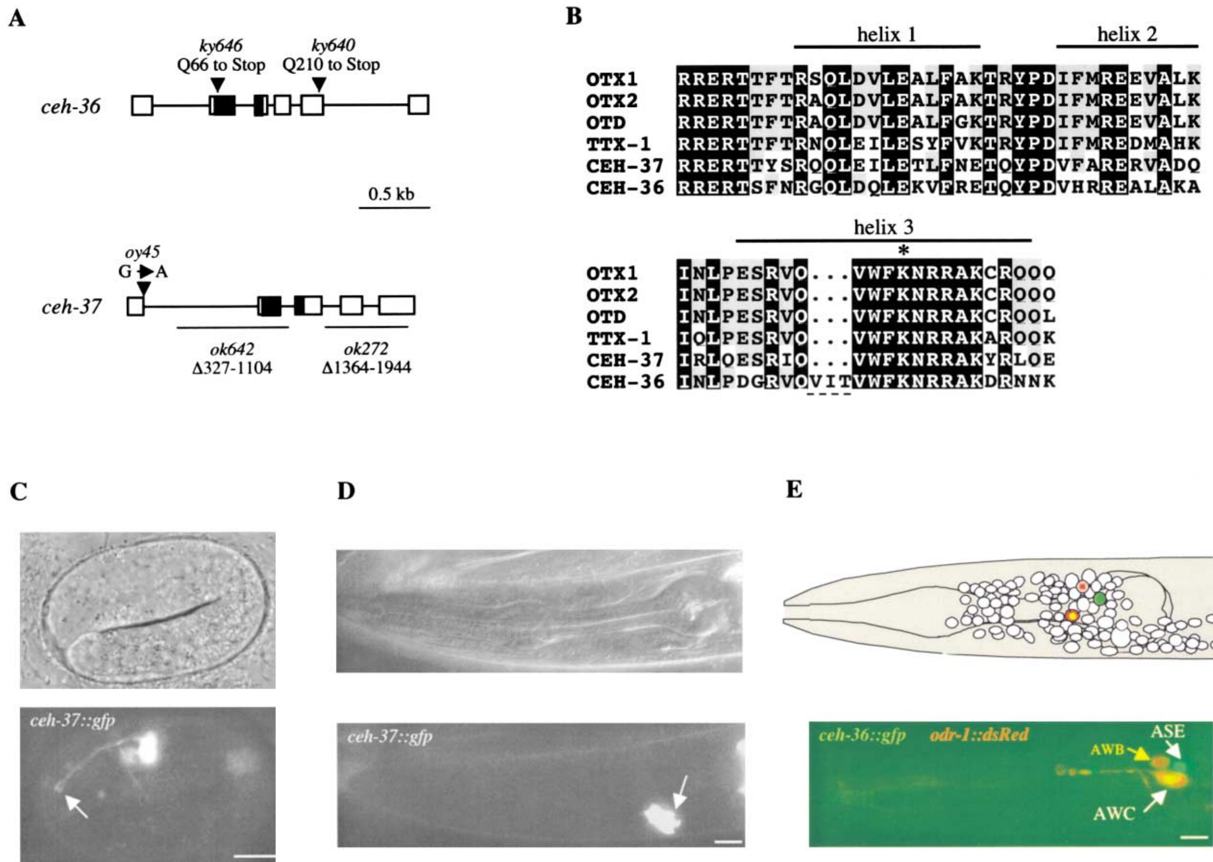

Figure 2. Genomic Structure and Expression Patterns of *ceh-36* and *ceh-37*

(A) *ceh-36* and *ceh-37* genomic sequences are adjacent on the cosmid C37E2. Shown are the genomic structures of both genes with the positions of the mutations and the extent of deletions marked. Filled boxes indicate the homeodomains.

(B) Alignment of the homeodomains of CEH-36, CEH-37, *C. elegans* TTX-1 (GI:25154683), rat OTX1 (GI:6981313), rat OTX2 (GI:27699028), and *Drosophila* OTD (GI:8311). The alignment was performed using the ClustalW algorithm (Higgins et al., 1996). Identical residues are boxed in black; conserved residues are boxed in gray. The positions of the helices are overlined. The asterisk indicates the characteristic Lys at position 9 of the third helix. The three amino acid insertion in the third helix of CEH-36 is marked with a dashed underline.

(C) A *ceh-37::gfp* fusion gene is expressed in the AWB neurons of 3-fold embryos. Arrow points to the characteristic forked cilia of an AWB neuron. The corresponding DIC image is shown at top.

(D) Expression of a *ceh-37::gfp* fusion gene is lost in head neurons and is maintained in the excretory cell (arrow) in adult animals. DIC image is at top.

(E) A *gfp*-tagged rescuing *ceh-36* transgene is expressed in the AWC and ASE neurons in adult animals (green). GFP is localized to the nuclei. These animals also express *odr-1::dsRed* strongly in the AWC and weakly in the AWB olfactory neurons (red). Schematic of the expression pattern is shown at top. Scale bar, 10 μm.

ceh-36 Is Required for the Specification of AWC and ASEL Identities

We next investigated the role of CEH-36 in the development and differentiation of the AWC and additional sensory neurons. Layered upon the basal AWC identity are mechanisms that distinguish one AWC neuron from its bilateral partner, and *str-2* acts as a marker for these distinct identities. Whereas the *str-2* olfactory receptor gene is expressed stochastically in either the left or the right AWC neuron (Troemel et al., 1999), *odr-1* is expressed in both AWC neurons (L'Etoile and Bargmann, 2000). Both AWC neurons respond to attractive odorants, such as benzaldehyde and isoamyl alcohol, but only the *str-2*^{ON} AWC neuron responds to the odorant butanone, and only the *str-2*^{OFF} AWC neuron mediates responses to 2,3-pentanedione (Wes and Bargmann, 2001). Expression of *odr-1::dsRed* was strongly reduced in both AWC neurons in *ceh-36* mutants (Table 2). *ceh-36* mutants also exhibited strong defects in their responses to all AWC-sensed odorants (Bargmann et al., 1993; Chou et al., 2001) (Figure 1D and data not shown), suggesting that *ceh-36* specifies the bilateral basal AWC identity rather than the unique identities of the left and right AWC neurons.

Like the AWC neurons, although the bilateral ASE neurons share a set of sensory functions and express genes in common, the ASEL and ASER neurons also mediate distinct sensory responses and exhibit distinct patterns of gene expression (Yu et al., 1997; Hobert et al., 1999; Pierce-Shimomura et al., 2001). In contrast to its role in the AWC neurons, mutations in *ceh-36* only weakly affected the expression of the bilaterally expressed gene

Table 1. Mutations in *ceh-37* Affect Expression of Genes in the AWB Olfactory Neurons

			% Expressing in the Indicated Number of Neurons		
Neuron Type Examined	Strain	Marker	None	One	Both
AWB	wild-type	*str-1::gfp*	0	0	100
	ceh-37(oy45)	*str-1::gfp*	15	57	28
	ceh-37(ok642)	*str-1::gfp*	37	43	18
	ceh-37(ok272)	*str-1::gfp*	13	41	46
	wild-type	*odr-1::dsRed*	0	0	100
	ceh-37(oy45)	*odr-1::dsRed*[a]	43	55	2
	ceh-37(ok272)	*odr-1::dsRed*[a]	37	47	16
	wild-type	*lim-4::gfp*	0	0	100
	ceh-37(oy45)	*lim-4::gfp*	16	50	33
	ceh-37(ok642)	*lim-4::gfp*	27	46	27
	ceh-37(ok272)	*lim-4::gfp*	8	45	48
ADF	wild-type	Ex[*T08G3.3::gfp*]	2	6	92
	ceh-37(oy45)	Ex[*T08G3.3::gfp*]	2	55	44
ASEL	wild-type	*gcy-7::gfp*	0	98	2
	ceh-37(oy45)	*gcy-7::gfp*	4	96	0
ASER	wild-type	*gcy-5::gfp*	0	100	0
	ceh-37(oy45)	*gcy-5::gfp*	0	100	0
	ceh-37(ok272)	*gcy-5::gfp*	0	100	0
AWC	wild-type	*str-2::gfp*[b]	0	100	0
	ceh-37(oy45)	*str-2::gfp*	0	98	2
	ceh-37(ok272)	*str-2::gfp*	1	99	0
AFD	wild-type	*gcy-8::gfp*	0	0	100
	ceh-37(oy45)	*gcy-8::gfp*	0	17	83
	ceh-37(ok642)	*gcy-8::gfp*	0	1	99

The expression of stably integrated transgenes were examined except for *T08G3.3::gfp* which was present in an extrachromosomal array. $n > 45$ for each. Adult animals grown at 25°C were examined.
[a] *odr-1::dsRed* expression was unaffected in the AWC neurons.
[b] *str-2::gfp* is expressed stochastically in either the left or the right AWC neuron in wild-type animals.

flp-6 (Li et al., 1999a, 1999b) in both the ASEL and ASER neurons (Table 2). However, expression of the ASEL-specific gene *gcy-7* (Yu et al., 1997) was abolished in *ceh-36* mutants (Table 2). The LIM homeobox gene *lim-6* is asymmetrically expressed in the ASEL neuron and represses expression of the ASER-specific marker *gcy-5*

Table 2. Mutations in *ceh-36* Affect Expression of Genes in the AWC Olfactory and ASE Chemosensory Neurons

			% Expressing in the Indicated Number of Neurons		
Neuron Type Examined	Strain	Marker	None	One	Both
AWC	wild-type	*str-2::gfp*[a]	1	99	0
	ceh-36(ky646)	*str-2::gfp*	99	1	0
	ceh-36(ky640)	*str-2::gfp*	100	0	0
	wild-type	*odr-1::dsRed*[b]	0	0	100
	ceh-36(ky646)	*odr-1::dsRed*	72	22	6
	ceh-36(ky640)	*odr-1::dsRed*	98	2	0
ASE	wild-type	*flp-6::gfp*	0	0	100
	ceh-36(ky646)	*flp-6::gfp*	0	19	81
ASEL	wild-type	*gcy-7::gfp*	0	98	2
	ceh-36(ky646)	*gcy-7::gfp*	100	0	0
	ceh-36(ky640)	*gcy-7::gfp*	100	0	0
	wild-type	*lim-6::gfp*	0	100	0
	ceh-36(ky646)	*lim-6::gfp*	9	91	0
	ceh-36(ky640)	*lim-6::gfp*	25	75	0
ASER	wild-type	*gcy-5::gfp*	0	100	0
	ceh-36(ky646)	*gcy-5::gfp*	0	95	5
	ceh-36(ky640)	*gcy-5::gfp*	0	88	12
AWB	wild-type	*str-1::gfp*	0	0	100
	ceh-36(ky646)	*str-1::gfp*	0	0	100
AFD	wild-type	*gcy-8::gfp*	0	0	100
	ceh-36(ky646)	*gcy-8::gfp*	0	0	100

The expression of stably integrated transgenes were examined in all cases. $n > 45$ for each. Adult animals grown at 25°C were examined.
[a] *str-2::gfp* is expressed stochastically in either the left or the right AWC neuron in wild-type animals.
[b] *odr-1::dsRed* is expressed strongly in the AWC and weakly in the AWB neurons in wild-type animals. Expression in the AWC neurons at levels less than in the AWB neurons was scored as loss of expression.

(Yu et al., 1997; Hobert et al., 1999). Expression of *lim-6* was also weakly affected in *ceh-36* alleles, and we observed ectopic expression of *gcy-5* in the ASEL neurons probably as a consequence of reduced *lim-6* expression. *gcy-5* expression in the ASER neurons was unaltered (Table 2). Thus, although *ceh-36* is required to specify the basal identities of the AWC neurons, in the ASE neurons, *ceh-36* acts primarily to diversify ASEL and ASER identities. *ceh-36* mutants did not exhibit defects in the expression of AWA, AWB, and AFD sensory neuron-specific markers and responded normally to additional sensory stimuli (Figure 1D, Table 2, and data not shown).

ceh-37 Is Expressed Transiently in the AWB Neurons, while *ceh-36* Expression Is Maintained in the AWC and ASE Neurons

To determine the spatial and temporal expression patterns of *ceh-37* and *ceh-36*, we examined the expression of reporter fusion genes. Animals transgenic for a rescuing *ceh-37* genomic fragment tagged with the Myc epitope expressed Myc in multiple cells prior to the comma stage of embryogenesis (see Supplemental Figure S2A). Interestingly, while the early expression of most *Otx* genes, including *ttx-1*, has been shown to be restricted to the anterior (Finkelstein and Boncinelli, 1994; Simeone, 1998; Hirth and Reichert, 1999; Klein and Li, 1999; Acampora et al., 2000; Satterlee et al., 2001), we also observed expression of *ceh-37::myc* in two cells in the posterior (see Supplemental Figure S2B). By the 3-fold stage, expression was largely restricted to the head region, with expression observed in neuronal and additional nonneuronal cells. Since individual neuronal cells were difficult to identify by position in late embryonic stages, we instead examined the expression of a *ceh-37* promoter*::gfp* fusion gene, which would enable us to identify neuron types based on morphology. The expression pattern of *ceh-37::gfp* was similar to that of *ceh-37::myc*. A pair of neuronal cells that showed strong expression of *gfp* was identified as the AWB neurons based on their characteristic ciliary morphology (Figure 2C). Expression in the AWB and additional neurons was transient such that expression was no longer observed by the early L1 larval stages. However, expression in nonneuronal cells, including the excretory cell and intestine, was maintained through adult stages (Figure 2D).

We next examined the expression pattern of a *ceh-36::gfp* transgene. Sequences encoding GFP were fused to the C terminus of *ceh-36* coding sequences. This *gfp*-tagged fusion gene rescued the AWC gene expression phenotype of *ceh-36* mutants (data not shown). Like *ceh-37*, *ceh-36::gfp* was also broadly expressed during early embryonic development (see Supplemental Figure S2C). *ceh-36* expression became restricted to the AWC and ASE chemosensory neuron nuclei during late embryonic stages (see Supplemental Figure S2D) and was maintained through the adult stage (Figure 2E). Interestingly, although the gene expression defects in *ceh-36* mutants were primarily restricted to the ASEL neurons, we observed *ceh-36::gfp* expression in both the ASEL and ASER neurons.

Subsets of *Otx* Genes Can Functionally Substitute for Each Other in the Specification of AWB, AFD, and AWC Neuron Identities

We investigated whether the *C. elegans Otx* genes and rat *Otx1* can functionally substitute for each other in the specification of neuronal identities. We first expressed *Otx* cDNAs under the *ttx-1* promoter in *ttx-1(p767)* mutants and examined AFD-specific gene expression. Expression of either the *ttx-1* or *ceh-37* cDNA fully rescued the *gcy-8::gfp* gene expression defects of *ttx-1(p767)* mutants (Figure 3A). As demonstrated previously, rat *Otx1* compensated poorly for the loss of *ttx-1* function (Satterlee et al., 2001). In addition, *ceh-36* failed to rescue the *gcy-8::gfp* expression phenotype. The CEH-36 HD includes a three residue insertion (VIT) that is absent in other members of the OTX family. Interestingly, deletion of these three amino acids [CEH-36(-VIT)] resulted in a significant increase in the ability of CEH-36 to rescue the *gcy-8::gfp* gene expression defects in the AFD neurons (Figure 3A).

TTX-1 maintains its own expression and also regulates the expression of target genes, such as *gcy-8* (Satterlee et al., 2001). We asked whether the failure of CEH-36 and OTX1 to regulate *gcy-8* expression results from a failure to maintain expression of these proteins from the *ttx-1* promoter-driven transgenes. However, *ttx-1::gfp* expression was maintained similarly in *ttx-1(p767)* mutants expressing either *ttx-1::ttx-1* or *ttx-1::ceh-36* (Figure 3A), suggesting that CEH-36 is able to substitute for TTX-1 in autoregulation but not in the expression of additional AFD-specific genes. Multiple independent transgenic lines generated upon microinjection of varying concentrations of *ceh-36* or *Otx1* cDNAs exhibited a similar failure to rescue the *gcy-8* expression defect. Taken together with the observation that CEH-36(-VIT) showed significant rescue of the *ttx-1* mutant phenotype, this suggests that the inability of CEH-36 to fully substitute for TTX-1 is likely not simply due to inappropriate levels of expression but due to differences in CEH-36 and TTX-1 protein functions.

We also expressed the *Otx* cDNAs under the *ceh-36* promoter in *ceh-36(ky646)* mutants and determined whether AWC-specific gene expression and sensory functions were rescued. All *Otx* cDNAs significantly rescued both AWC-specific gene expression and AWC-mediated behavior (Figures 3B and 3C), indicating that these genes are able to substitute for *ceh-36* in the specification of AWC identity.

We next expressed *ceh-36*, *ttx-1*, *ceh-37*, and rat *Otx1* cDNAs under the *osm-6* promoter, which drives expression in all ciliated neurons in *C. elegans*, including the AWB neurons (Collet et al., 1998). Expression of all *Otx* cDNAs resulted in significant rescue of *str-1::gfp* expression in *ceh-37(oy45)* mutants (Figure 3D). This function was restricted to *Otx*-type homeobox genes and was dependent upon the homeodomain. We were unable to determine whether the sensory behaviors mediated by the AWB neurons and AWB-specific morphology were restored because of the partially penetrant phenotype of *ceh-37* null mutants.

Taken together, these results indicate that while all *Otx* genes can functionally substitute for each other in the specification of AWB and AWC neuronal identities,

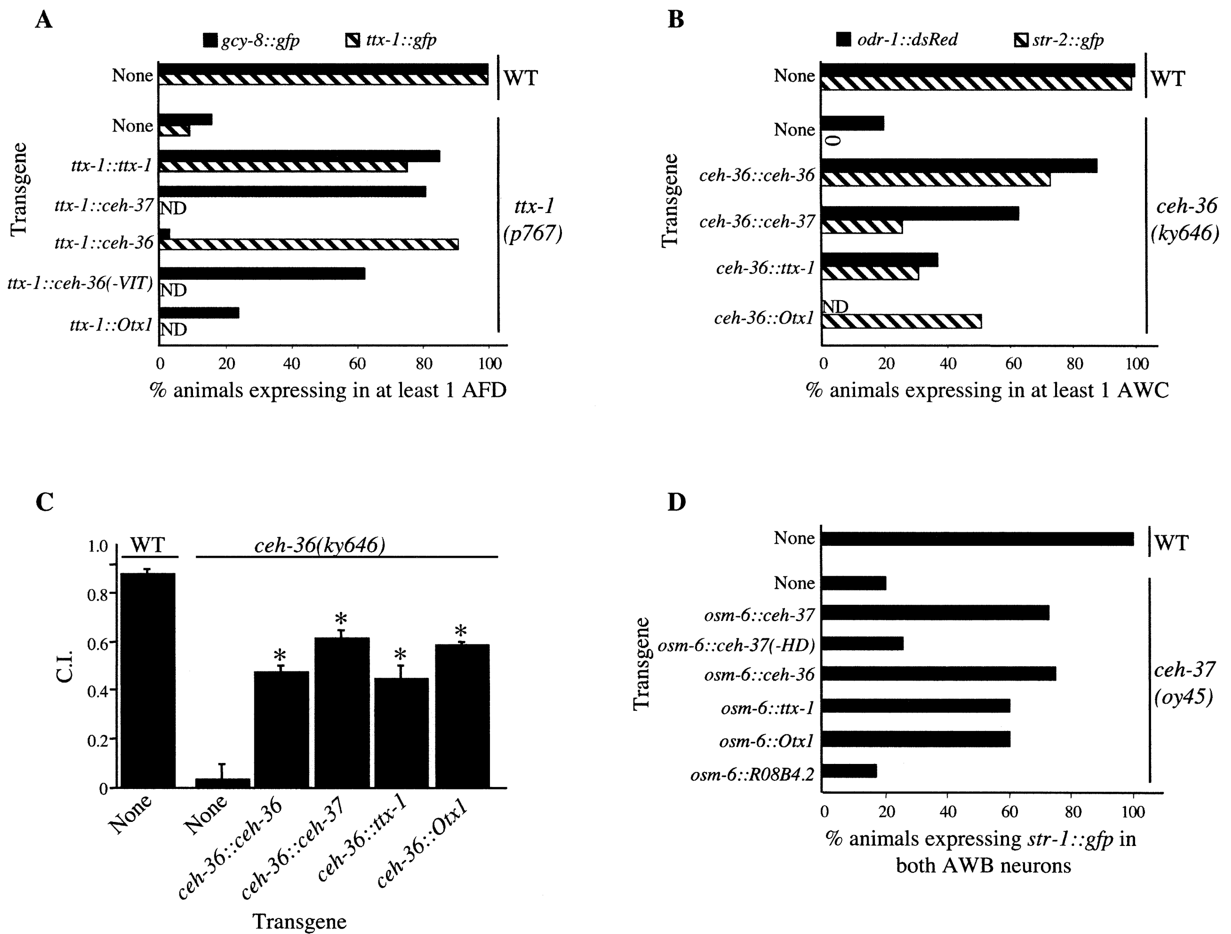

Figure 3. Some *Otx* Genes Can Functionally Complement Each Other in the Specification of AFD, AWC, and AWB Identities

(A) Percentage of animals of the indicated genotypes expressing either *gcy-8::gfp* (black bars) or *ttx-1::gfp* (hatched bars) in at least one AFD neuron. *gcy-8::gfp* was stably integrated in the genome; *ttx-1::gfp* was present in extrachromosomal arrays in a *rol-9(sc148)* strain. Transgenes were introduced into the same Ex[*ttx-1::gfp*] carrying line. Numbers shown are from one to two independent transgenic lines each. Strains were scored blindly. n > 40–130 for lines expressing *gcy-8::gfp*; n > 35 for lines expressing *ttx-1::gfp*.

(B) Percentage of animals expressing stably integrated *odr-1::dsRed* (black bars) or *str-2::gfp* (hatched bars) in the AWC neurons. *odr-1::dsRed* expression was scored as indicated in Table 3. Numbers shown are from one to three independent transgenic lines each. n > 45 for each.

(C) Responses of animals of the indicated genotypes to a point source of 1 μl of 1:200 dilution of benzaldehyde. The *ceh-36(ky646)* strain used carries stably integrated copies of the *odr-1::dsRed* fusion gene. Each data point represents the mean of at least two independent assays using ~100 animals in each assay. At least two independent lines were examined for each transgene. *Indicates responses that are different from those of *ceh-36(ky646)* at $p < 0.001$. C.I., chemotaxis index. Error equals the SEM.

(D) Percentage of animals of the indicated genotypes expressing *str-1::gfp* from stably integrated arrays in both AWB neurons. R08B4.2 encodes a non-*Otx* member of the paired class homeodomain family. Numbers are from one or two independent transgenic lines each. n > 50 for each. Transgenes were injected at 10 or 30 ng/μl with coinjection markers (see Experimental Procedures). "None" refers to the appropriate strain carrying the coinjection marker. Adult animals grown at 25°C were examined. ND, not done.

a subset of these genes may have acquired divergent functions. Thus, the distinct phenotypes of the *Otx* gene mutants in *C. elegans* may result from divergence in both gene function and gene regulatory mechanisms.

TTX-1 and CEH-37 Act via LIM-4 to Regulate AWB Neuron Identity

Since expression of both *ceh-37* and *ttx-1* in the AWB neurons restored AWB development, we next asked whether these genes act by regulating the same downstream target(s), or whether they activate distinct downstream developmental pathways that result in phenotypically equivalent developmental outcomes.

Mutations in *ceh-37* downregulate *lim-4* expression, indicating that CEH-37 promotes AWB identity by regulating the expression of *lim-4*. Expression of both the *ceh-37* or the *ttx-1* cDNA under the *osm-6* promoter in *ceh-37(oy45)* mutants restored *lim-4::gfp* expression in the AWB neurons to similar levels (Table 3). Moreover, both *ceh-37* and *ttx-1* required LIM-4 function to restore *str-1* expression, since expression of either *osm-6::ceh-37* or *osm-6::ttx-1* was not able to restore *str-1* expression in *ceh-37 lim-4* double mutants (Table 3). These results indicate that TTX-1 compensates for CEH-37 function in the AWB neurons by activating the same downstream target.

Table 3. CEH-37 and TTX-1 Act via LIM-4 to Regulate AWB Identity

Strain	Marker	% Animals Expressing in Both AWB Neurons	n
Wild-type	*lim-4::gfp*	100	83
ceh-37(oy45)	*lim-4::gfp*	33	153
ceh-37(oy45); Ex[*osm-6::ceh-37*]	*lim-4::gfp*	78	37
ceh-37(oy45); Ex[*osm-6::ttx-1*]	*lim-4::gfp*	65	92
Wild-type	*str-1::gfp*	100	$>$100
ceh-37 (ok272) lim-4(ky403)	*str-1::gfp*	0	53
ceh-37 (ok272) lim-4(ky403); Ex[*osm-6::ceh-37*]	*str-1::gfp*	0	48
ceh-37 (ok272) lim-4(ky403); Ex[*osm-6::ttx-1*]	*str-1::gfp*	0	72

lim-4::gfp and *str-1::gfp* were stably integrated in the genome. pRF4 was used as the coinjection marker. Numbers shown are from one to two independent transgenic lines each.

Ectopic Expression of *Otx*-like Genes Promotes AFD or AWC but Not AWB-like Fates in Nonoverlapping Cell Types

Our misexpression experiments suggest that although an *Otx* gene is capable of specifying multiple neuronal identities, the cellular context of expression constrains the specific identity adopted. To explore how misexpression of *Otx* genes promotes different cell identities in different cell types, we examined the consequences of ectopic expression of *Otx* genes driven under the *osm-6* promoter.

osm-6::Otx genes were introduced into animals carrying stably integrated copies of the AFD marker *gcy-8::gfp* and the AWC marker *odr-1::dsRed*. We previously showed that misexpression of *osm-6::ttx-1* was sufficient to confer an AFD-like fate onto a subset of ciliated sensory neurons (Satterlee et al., 2001). Expression of *osm-6::ceh-36* and *osm-6::ceh-37* but not *osm-6::Otx1* also resulted in ectopic expression of *gcy-8::gfp* (Figures 4B and 4C, and data not shown). The AWC markers *str-2::gfp* and *odr-1::dsRed* were also misexpressed in a subset of sensory neurons upon misexpression of any of the four *Otx* genes (Figures 4B and 4C, and data not shown). In contrast, misexpression of the AWB markers *str-1::gfp* or *lim-4::gfp* was never observed in transgenic *osm-6::Otx* strains.

We investigated whether AFD- and AWC-specific genes were misexpressed in overlapping or nonoverlapping sets of cells. As the large numbers of misexpressing cells in the head made it difficult to examine single neuron types, we focused on the four sensory neurons of the phasmid organs in the tail. Ninety-nine percent of transformed phasmid sensory neurons examined ($n = 280$) (Figure 4D) expressed either the AFD or AWC marker but not both, suggesting that these neurons adopted either AFD- or AWC-like features but did not adopt a hybrid identity.

Unlike the AWB, AWC, and AFD neurons, the cellular context of a phasmid neuron did not appear to constrain the sensory neuron identity adopted upon misexpression of a specific *Otx* gene. We found that a given phasmid neuron could adopt either the AWC or AFD cell identities upon misexpression of an *Otx* gene. Interestingly, in this cellular context, different *Otx* genes preferentially promoted either the AFD or AWC identities. Expression of *osm-6::ceh-36* resulted in a larger number of transformed phasmid cells expressing the AWC marker, whereas expression of either *osm-6::ceh-37* or *osm-6::ttx-1* resulted in a larger number of phasmid neurons misexpressing the AFD marker (Figure 4E). Similar differences were observed in the amphid neurons (A.L. and P.S., unpublished data). The relative differences in the abilities of these *Otx* genes to promote distinct cellular characteristics were maintained in multiple independent transgenic lines derived from injecting higher or lower concentrations of the *Otx* genes (data not shown). These results reveal that *Otx* genes promote different cell identities to different extents and that cell types, such as the phasmid neurons, are not restricted in their choice of cellular identity upon misexpression of an *Otx* gene.

Discussion

The precise generation of multiple neuronal subtypes is critical for the remarkably complex functions of the nervous system. However, the mechanisms by which diverse neuronal subtypes are specified have not been fully explored. In vertebrates, different members of the basic-helix-loop-helix and LIM homeodomain protein families have been shown to regulate different motor neuron subtype identities in the spinal cord (Bertrand et al., 2002; Shirasaki and Pfaff, 2002), but whether similar principles apply to other neuron types is largely unknown. We have shown that three members of the *Otx* gene family specify different sensory neuronal subtypes in the *C. elegans* nervous system, thereby increasing functional complexity. Taken together with previous findings, these results suggest that a basic principle by which cellular diversity may be achieved in the vertebrate and invertebrate nervous systems is via the partly diverged functions of homologous genes acting in developmentally defined cellular contexts.

Members of the *Otx* Gene Family Specify Distinct Neuronal Subtype Identities via Different Mechanisms

The *ceh-36, ceh-37*, and *ttx-1 Otx* genes share both similarities and differences in their mechanisms of action. Both *ceh-36* and *ttx-1* are expressed through the adult stages in the AWC and AFD neurons. We previously showed that in *ttx-1* mutants, AFD-specific gene expression is retained in larvae but lost in adults (Satterlee et al., 2001), suggesting that TTX-1 is required for maintenance but not initiation of AFD-specific gene expression. In *ceh-36* mutants, however, AWC-specific

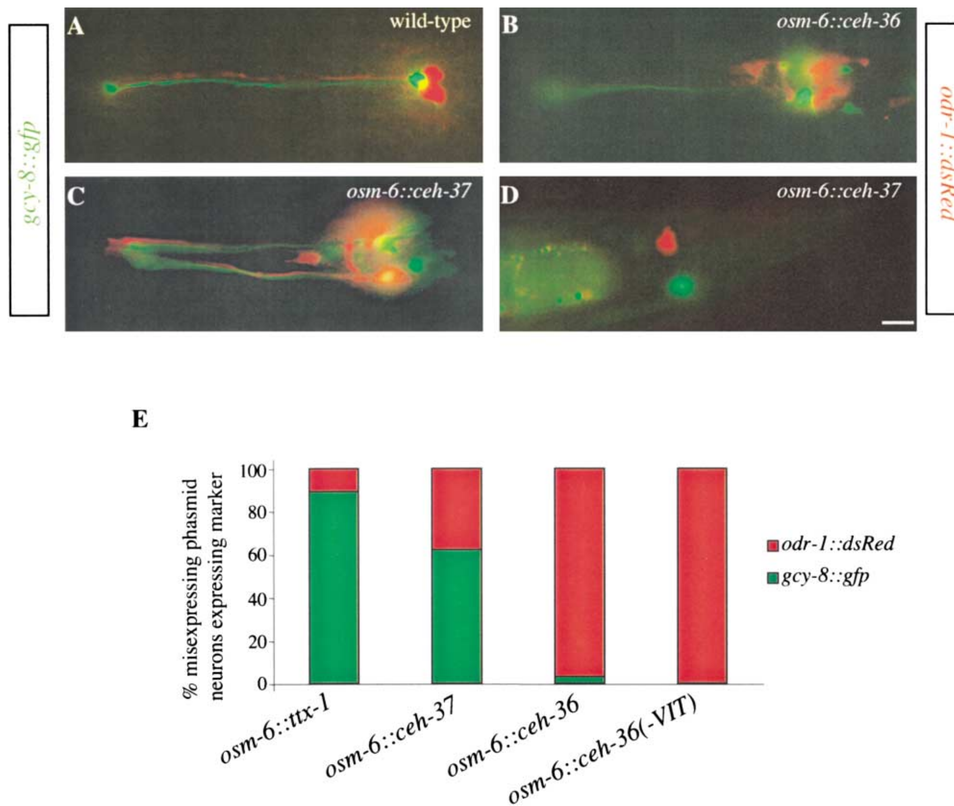

Figure 4. Misexpression of Different *Otx* Genes Causes Ectopic Expression of AFD- and AWC-Specific Markers to Different Extents

(A) Expression of stably integrated *gcy-8::gfp* and *odr-1::dsRed* transgenes in wild-type animals.

(B and C) Ectopic expression of markers in the head of a transgenic animal expressing *osm-6::ceh-36* (B) and *osm-6::ceh-37* (C).

(D) Representative example of ectopic expression of markers in the phasmid neurons upon *osm-6::ceh-37* expression. On average, 1.0 phasmid neurons per transgenic animal misexpressed *gcy-8::gfp* and 0.6 phasmid neurons per transgenic animal misexpressed *odr-1::dsRed* (n = 216 phasmid neurons examined). Anterior is at left; scale bar, 10 μm.

(E) Percentage of transformed phasmid neurons expressing either *gcy-8::gfp* or *odr-1::dsRed* upon expression of the indicated transgenes. n > 35 for each; one to two transgenic lines each. A given marker was misexpressed in up to four phasmid neurons per animal.

gene expression appears to be lost at all postembryonic stages, implicating CEH-36 in initiation and possibly also in maintenance of gene expression. In the ASE neurons, *ceh-36* acts at a step in the developmental hierarchy that is distinct from its role in the AWC neurons. *ceh-36* is required to diversify the fate of the ASEL neurons from the ASER neurons but not to regulate general or basal ASE characteristics. CEH-36 has recently been shown to interact with additional transcription factors to mediate its functions in the ASE neurons (Chang et al., 2003). It is unclear whether TTX-1 and CEH-36 regulate the expression of neuron-specific terminal differentiation genes directly or indirectly (Sheng et al., 1997; Niimi et al., 1999; Livesey et al., 2000; Gaudet and Mango, 2002). TTX-1 regulates the expression of the *ceh-14* LIM homeobox gene required for partial differentiation of AFD morphology (Cassata et al., 2000; Satterlee et al., 2001), suggesting that TTX-1 may mediate its functions in part by regulating multiple downstream transcription factors. CEH-36 may act similarly in the AWC neurons.

In contrast to the expression patterns of *ceh-36* and *ttx-1*, *ceh-37* is expressed only transiently in the AWB neurons. A simple model for CEH-37 function suggests that embryonic expression of *ceh-37* in the AWB neurons is sufficient to trigger *lim-4* expression, which then maintains its expression via autoregulation and also regulates AWB identity. A similar temporal cascade of transcription factors was previously shown to be required for the specification of identity of the AWA olfactory neurons and the AIY interneurons (Altun-Gultekin et al., 2001; Sarafi-Reinach et al., 2001). Although we have not further investigated the role of CEH-37 in the specification of ADF neuron identity, we did not observe *ceh-37* expression in this neuron type in adult animals, suggesting that, as in the AWB neurons, CEH-37 may also act transiently in the ADF neurons. Thus, *Otx* genes can act at different steps in the developmental hierarchies required for the acquisition of sensory neuronal identities.

Despite these dissimilarities in their modes of action, the overall roles of the *Otx*-like genes in the specification of sensory neuron identities are quite similar. In all three mutants, a set of affected neurons fail to express tested neuron-specific characteristics and appear to retain only "generic" sensory neuron features. In *ttx-1* mutants, although a subset of affected AFD neurons misexpress the AWC marker *str-2*, these neurons do not express

additional AWC-specific genes and do not adopt an AWC-like sensory morphology (Perkins et al., 1986; Sarterlee et al., 2001). The AFD neurons instead exhibit undifferentiated sensory ciliary morphology. Similarly, the AWC neurons in *ceh-36* mutants also appear to retain a bipolar sensory morphology but do not exhibit features characteristic of a given sensory neuron type. The role of *ceh-37* in the AWB neurons is particularly informative. CEH-37 appears to be required to both regulate *lim-4* expression and to confer the default AWC fate. Thus, in *ceh-37* mutants, the AWB neurons express neither an AWB nor an AWC identity and instead express apparently undifferentiated sensory neuron characteristics. Taken together, *Otx*-like genes appear to be required for conferring neuron-specific identities in all three neuron types, albeit via different mechanisms.

The Distinct Functions of *Otx* Genes Are Mediated by Divergence in Spatiotemporal Expression Patterns and by the Acquisition of New Functions

In mice, both *Otx2* and *otd* can substitute for *Otx1* for the majority of *Otx1*-mediated functions (Acampora et al., 1998a, 1999). However, the defects in the lateral semicircular canal of the inner ear present in *Otx1* mutant mice are not rescued (Acampora et al., 1998a, 1999; Morsli et al., 1999). This result has led to the hypothesis that, although the distinct phenotypes of the *Otx1* and *Otx2* mutant mice are largely a consequence of divergence in the regulation of spatiotemporal gene expression as opposed to divergence in gene functions, OTX1 may have acquired additional functions (Simeone et al., 1993; Li and Noll, 1994; Acampora and Simeone, 1999; Morsli et al., 1999; Acampora et al., 2000). Similarly, our results indicate that while all three *Otx*-like genes appear to be able to promote AWB and AWC neuronal characteristics, only *ceh-37* is capable of substituting for *ttx-1* function in the AFD neurons. Divergence in the 3′ UTRs of *Otx1* and *Otx2* has been shown to result in differential translation of these gene transcripts in the visceral endoderm and epiblast (Acampora et al., 1998b, 2001; Boyl et al., 2001). Although similar divergence in posttranscriptional regulatory mechanisms or differences in protein threshold requirements may play a role in the inability of *ceh-36* to fully substitute for the *ttx-1* mutant phenotype, the partial rescue observed upon expression of the mutant CEH-36(-VIT) protein suggests that divergence in protein function in the context of the AFD neurons is likely to be critical. Thus, as in mammals, divergence in both OTX protein function as well as gene expression patterns may account for the distinct mutant phenotypes of the *Otx* genes in *C. elegans*. These differences may result from different rates of evolutionary change in the regulatory and coding sequences of these genes which presumably arose via gene duplication (Wagner, 2000).

Knowledge of the downstream genetic pathways regulated by *Otx* genes has allowed us to investigate the operational basis for the observed ability of the *Otx* genes to functionally substitute for each other. Functional equivalence in OTX activity has been defined primarily on the basis of rescued mutant phenotypes. However, in the absence of knowledge regarding the underlying mechanisms, it has remained unclear whether the ability of an *Otx* gene to substitute for another reflects equivalence in protein functions. Expression of the human *Otx* genes in *Drosophila* has been shown to regulate a similar set of targets as *Drosophila otd* (Nagao et al., 1998; Montalta-He et al., 2002). We have now shown that rescue of the AWB specification defect by both *ceh-37* and *ttx-1* requires *lim-4*, indicating that phenotypic equivalence arises as a consequence of activation of the same downstream genetic target by both OTX proteins. Thus, the phenotypic equivalence in CEH-37 and TTX-1 functions appears to be a consequence of equivalence in protein activity in the AWB neurons.

Acquisition of Specific Neuronal Characteristics upon Expression of an *Otx* Gene Is Dependent Both on the Cellular Context and the Identity of the *Otx* Gene

All three *Otx*-like genes can drive ectopic expression of AWC and AFD but not AWB-specific markers when misexpressed in other sensory neurons. Despite this functional flexibility, expression of subsets of the three *Otx* genes in the AWB, AWC, or AFD neurons preferentially promote the respective neuronal identities. We observed occasional faint misexpression of the AFD marker *gcy-8::gfp* in the AWC neurons upon overexpression of *Otx* genes, including *ceh-36* (A.L. and P.S., unpublished data), but similar misexpression of AWC markers was not observed in the AFD neurons. This result indicates that the cellular context of these neuron types constrains, although may not fully restrict, the adopted identity upon expression of an *Otx* gene.

Interestingly, expression of the *Otx* genes in the phasmid neurons uncovers intrinsic preferences of the OTX proteins for promoting distinct cell fates. In the phasmid neurons, the cellular context does not appear to constrain the identity selected upon misexpression of an *Otx* gene, such that an individual phasmid neuron may adopt either AWC or AFD cellular features. Instead, the identity chosen is partly dictated by the *Otx* gene expressed. Expression of *ceh-36* appears to preferentially allow the expression of AWC markers, whereas expression of *ceh-37* or *ttx-1* preferentially directs the expression of AFD-specific markers. Although the ability of *ceh-36* to preferentially direct the AWC fate may be in part due to its relative inefficiency in directing the AFD fate, *ceh-37* and *ttx-1* appear to be capable of directing either the AWC or AFD fates when expressed in the AWC or AFD neurons, respectively. These results reveal differences in the intrinsic functions of these OTX proteins and suggest that phenotypic equivalence may arise partly as a consequence of restriction of function by the cellular context of expression.

Concluding Remarks

Our findings implicate the *Otx* genes in a relatively late step in the developmental hierarchies required for the generation of different sensory neuron types. *Otx* genes act to confer neuron subtype specific but not general sensory neuron characteristics onto individual neuron types. Late developmental roles have also been proposed for *Otx2* and the divergent *Otx*-like gene *Crx* in vertebrate retinal development and for the *otd* gene in

the morphological differentiation of *Drosophila* photoreceptors (Vandendries et al., 1996; Bovolenta et al., 1997; Furukawa et al., 1999; Martinez-Morales et al., 2001). Our results emphasize the importance of defining the context in which *Otx* as well as other genes function and underscore the necessity to investigate the functions of individual genes as components of broader genetic networks.

Experimental Procedures

Strains

Worms were grown using standard methods (Brenner, 1974). Strains were obtained from the *Caenorhabditis* Genetics Center unless noted otherwise. Strains carrying integrated transgenes were provided as indicated: *ntIs1* (*gcy-5::gfp*) (gift from S. Lockery; Yu et al., 1997); *otIs3* (*gcy-7::gfp*), *otIs114* (*lim-6::gfp*), *otIs125* (*flp-6::gfp*) (gift from O. Hobert; Yu et al., 1997; Hobert et al., 1999; Li et al., 1999b); *oyIs17* (*gcy-8::gfp*), *oyIs18* (*gcy-8::gfp*) (Satterlee et al., 2001); *kyIs104* (*str-1::gfp*), *kyIs140* (*str-2::gfp*) (Troemel et al., 1997, 1999).

Strains carrying the following stably integrated transgenes were generated as described (Satterlee et al., 2001): *oyIs44* (*odr-1::dsRed*) (L'Etoile and Bargmann, 2000), *oyIs35* (*lim-4::gfp*), and *oyIs46* (*ceh-37::myc*). *lin-15(+)* was used as the coinjection marker. Each integrated strain was backcrossed at least four times prior to analysis.

Isolation, Mapping, and Cloning of *ceh-37* and *ceh-36* Alleles

ceh-37(oy45) and *ceh-36* alleles were isolated from an EMS mutagenesis screen of *kyIs104* (*str-1::gfp*) and *kyIs140* (*str-2::gfp*), respectively, essentially as described previously (Lanjuin and Sengupta, 2002). *ceh-37(oy45)* was mapped with respect to polymorphisms and deficiencies to a small interval on LG X. The loss of *str-1::gfp* expression in *ceh-37(oy45)* animals was rescued by a single cosmid in this interval, C37E2 and the single ORF C37E2.5. *ceh-36(ky646)* and *ceh-36(ky640)* failed to complement for the loss of *str-2* expression phenotype and were mapped to LG X with respect to visible markers. The loss of *str-2::gfp* expression in *ceh-36* mutants was rescued by the single ORF C37E2.4. To identify the molecular nature of the lesions in *ceh-36* and *ceh-37* alleles, genomic sequences were amplified in multiple independent reactions and sequenced. The *ceh-37(oy45)* mutation results in a G to A transition at the 5′ splice donor site of the first intron. *ceh-37* transcripts amplified from *ceh-37(oy45)* animals included sequences from the first intron (data not shown); translation of this aberrantly spliced message is predicted to result in a mutant CEH-37 protein truncated at residue 34, thereby deleting the HD. No correctly spliced messages were identified.

Generation of Expression Constructs and Transgenic Animals

Reporter Gene Constructs

A *ceh-37* promoter::*gfp* fusion gene was generated by fusing ~3.2 kb of 5′ upstream sequences and sequences in the first exon encoding the first 17 amino acids in frame to *gfp*. A *myc*-tagged *ceh-37* expression construct was generated by replacing homeodomain residues in a rescuing full-length *ceh-37* genomic fragment with sequences encoding five repeats of the Myc epitope. *gfp*-tagged *ceh-36* was generated by fusing *gfp* coding sequences in frame just prior to the stop codon in a full-length rescuing *ceh-36* genomic fragment. All expression constructs were injected at 30 ng/μl with *lin-15(+)* as the coinjection marker and exhibited similar expression patterns, except that GFP-tagged and Myc-tagged proteins were localized to nuclei.

Misexpression Constructs

ceh-36 and *ceh-37* transcripts (including 144 and 302 bp 3′ UTR sequences, respectively) were amplified from an oligo-d(T) primed RT reaction using total worm RNA. Each cDNA was sequenced and inserted into a modified pPD95.77 cloning vector (gift from M. Colosimo; gift from A. Fire) containing the appropriate promoter sequences. *ttx-1* and *Otx1* cDNAs were generated as described (Satterlee et al., 2001).

For misexpression in the AWB and phasmid neurons, all *Otx* cDNAs were driven by the *osm-6* promoter (Collet et al., 1998) and injected at 30 ng/μl using pRF4 as the coinjection marker. For misexpression in the AWC neurons, all *Otx* cDNAs were driven by ~1.8 kb of *ceh-36* promoter sequences and injected at 30 ng/μl using *unc-122::gfp* as the coinjection marker (for rescue of AWC-mediated behaviors and *odr-1::dsRed* expression) or at 10 ng/μl using pRF4 as the coinjection marker (for rescue of *str-2::gfp* expression). For misexpression in the AFD neurons, *Otx* cDNAs were driven by the *ttx-1* promoter (Satterlee et al., 2001) and injected at 10 ng/μl using pRF4 or *T08G3.3::dsRed* as the coinjection markers. The *ttx-1::ceh-36(-VIT)* plasmid was derived from *ttx-1::ceh-36* using the QuikChange site-directed mutagenesis kit (Stratagene, CA). Transgenic lines generated by injection of misexpression constructs at higher or lower concentrations yielded essentially the same results.

Immunocytochemistry

oyIs46 embryos were fixed and permeabilized using a modified protocol (Chin-Sang et al., 1999). Mouse monoclonal anti-c-*myc* antibodies (Roche Applied Science, IN) were used as the primary antibodies at 10 μg/ml. Secondary goat anti-mouse IgG antibodies conjugated to rhodamine (Jackson Immunoresearch, PA) were used at a 1:25 dilution. Microscopy was carried out as previously described (Sarafi-Reinach and Sengupta, 2000).

Behavioral Assays

Chemosensory behavioral assays were performed as described (Bargmann and Horvitz, 1991; Bargmann et al., 1993; Troemel et al., 1997). All strains assayed were backcrossed a minimum of five times prior to behavioral analysis. Statistical analysis involving comparisons among multiple groups was performed using the Bonferroni-Dunn multiple comparisons procedure, with the significance level set at 5%.

Acknowledgments

We are grateful to Maura Berkeley and Laura Vivier for technical assistance; Alan Coulson for cosmids; the *Caenorhabditis* Genetics Center for strains; Andy Fire and Marc Colosimo for plasmids; Oliver Hobert and Tali Melkman for sharing unpublished information; Sarah Bauer Huang for assistance with the *str-2::gfp* screen; and the *C. elegans* Genome Sequencing and Knockout consortium for sequence information and alleles. We acknowledge helpful advice and comments from members of the Sengupta lab. We thank Oliver Hobert and members of the Sengupta lab for critical comments on the manuscript; and Marc Colosimo for assistance with sequence analysis. This work was supported in part by grants from the NIH (GM56223 to P.S.; DC04089 to C.I.B.) and the NSF (IBN 0129370 to P.S.). P.S. was a Packard Foundation Fellow. C.I.B. is an Investigator of the Howard Hughes Medical Institute. M.K.V. is an NSF predoctoral fellow.

Received: June 27, 2003
Revised: August 26, 2003
Accepted: August 26, 2003
Published: October 6, 2003

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