A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans*

Zeynep Altun-Gultekin¹, Yoshi Andachi², Ephraim L. Tsalik¹, David Pilgrim³, Yuji Kohara² and Oliver Hobert¹,*

¹Department of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
²Genome Biology Lab, Center for Genetic Resource Information, National Institute of Genetics, Mishima, Shizuoka, 411-8540, Japan
³Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

*Author for correspondence (e-mail: or38@columbia.edu)

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SUMMARY

The development of the nervous system requires the coordinated activity of a variety of regulatory factors that define the individual properties of specific neuronal subtypes. We report a regulatory cascade composed of three homeodomain proteins that act to define the properties of a specific interneuron class in the nematode *C. elegans*. We describe a set of differentiation markers characteristic for the AII interneuron class and show that the *ceh-10* paired-type and *ttx-3* LIM-type homeobox genes function to regulate all known subtype-specific features of the AII interneurons. In contrast, the acquisition of several pan-neuronal features is unaffected in *ceh-10* and *ttx-3* mutants, suggesting that the activity of these homeobox genes separates pan-neuronal from subtype-specific differentiation programs. The LIM homeobox gene *ttx-3* appears to play a central role in regulation of AII differentiation. Not only are all AII subtype characteristics lost in *ttx-3* mutants, but ectopic misexpression of *ttx-3* is also sufficient to induce AII-like features in a restricted set of neurons. One of the targets of *ceh-10* and *ttx-3* is a novel type of homeobox gene, *ceh-23*. We show that *ceh-23* is not required for the initial adoption of AII differentiation characteristics, but instead is required to maintain the expression of one defined AII differentiation feature. Finally, we demonstrate that the regulatory relationship between *ceh-10*, *ttx-3* and *ceh-23* is only partially conserved in other neurons in the nervous system. Our findings illustrate the complexity of transcriptional regulation in the nervous system and provide an example for the intricate interdependence of transcription factor action.

Key words: Homeobox, *C. elegans*, Neuronal differentiation

INTRODUCTION

Differential gene expression patterns are a characteristic feature of individual cell types within the nervous system. Complex patterns of tightly controlled gene regulatory events are conferred by transcription factors that act at a variety of different stages during neuronal development. Some transcription factors act as early neuronal fate determinants such that their disruption leads to the adoption of distinct, non-neuronal cell fates. Other transcription factors function later in development to regionalize the nervous system, while the subsequent activity of yet other transcription factors determines specific subtype characteristics of neurons (Anderson and Jan, 1997; Bang and Goulding, 1996; Jurata et al., 2000). Transcription factors that act at distinct steps in the developmental history of a neuron most likely function in hierarchical cascades of gene regulation, in which early acting transcription factors turn on the expression of later acting transcription factors. The hierarchical activation of transcription factors presumably results in a progressive restriction in the developmental potential of a neuron, eventually leading to the acquisition of its terminal differentiation features. Transcriptional regulatory cascades have, for example, been described for neurogenic events involving bHLH proteins in vertebrates and flies (Anderson and Jan, 1997), cell fate determination in the vertebrate spinal cord (Edlund and Jessell, 1999; Tanabe et al., 1998), and cell subtype specification of a class of motoneurons (Baum et al., 1999) and touch sensory neurons (Mitani et al., 1993) in the nematode *C. elegans*.

A prominent class of transcription factors that acts to determine terminal differentiation characteristics of neurons is the LIM homeobox class of regulatory factors (Hobert and Westphal, 2000). In the mouse, *Isll* is required for early aspects of motoneuron differentiation (Pfaff et al., 1996), while later acting Lhx genes, such as *Lhx3* or *Lhx4* define the identity of specific subtypes of motoneurons in the spinal cord (Sharma et al., 1998; Tsuchida et al., 1994). *Lim1* and *Lmx1b* coordinately direct axonal outgrowth
patterns in vertebrate limbs (Kania et al., 2000). In Drosophila, genetic and neuroanatomical studies on apterous, islet and lim3 have revealed their involvement in several aspects of neuronal differentiation, including axon fasciculation, pathfinding and neurotransmitter choice (Lundgren et al., 1995; Thor et al., 1999; Thor and Thomas, 1997). As in vertebrates, combinatorial expression of Lhx genes in Drosophila determines motoneuron differentiation and axonal targeting choices in the ventral nerve cord (Thor et al., 1999).

As revealed by the genome sequencing project, C. elegans contains seven Lhx genes, which are expressed in largely non-overlapping sets of neurons (Hobert and Westphal, 2000). mec-3, one of the founding members of the Lhx gene family, represents the end-point in a cascade of transcriptional regulatory factors that determines the fate of touch sensory neurons (Mitani et al., 1993; Way and Chalfie, 1988). mec-3 directly regulates the transcription of genes required for touch sensory neuron function such as the mec-7 ß-tubulin gene and the mec-4 ion channel gene (Duggan et al., 1998). The lin-11, lim-6 and ceh-14 genes act to control as yet undefined subtype characteristics of specific neurons and other tissue types (Cassata et al., 2000; Freyd et al., 1990; Hobert et al., 1998; Hobert et al., 1999), while the lin-4 gene functions as a binary cell-type switch in a class of odorsensory neurons (Sagasti et al., 1999).

There are three central aspects of Lhx gene function that remain poorly understood in any system. First, the precise nature of cellular defects evoked by loss of Lhx protein function is often unclear owing to the paucity of specific cell fate and neuroanatomical markers. Second, with a few exceptions (Benveniste et al., 1998; Duggan et al., 1998), target genes of Lhx proteins in the nervous system have remained largely elusive. Finally, the nature of upstream regulators of Lhx genes and hence the integration of Lhx gene function into larger transcriptional regulatory cascades or networks is little understood.

We address these key questions for the C. elegans Lhx gene ttx-3. We have previously shown that ttx-3 mutant animals display defects in thermoregulatory behavior that mimic the defects seen upon ablation of a class of interneurons in the thermoregulatory neuronal circuit, the AIY interneuron class (Hobert et al., 1997). The expression of ttx-3 in the AIY interneurons of wild-type animals hence suggested that ttx-3 is required for the AIY neurons to function appropriately (Hobert et al., 1997). We now show that within the AIY interneurons ttx-3 is part of a transcriptional regulatory cascade, which involves two other homeobox genes, ceh-10 and ceh-23. We demonstrate that ttx-3 is not required for the adoption of pan-neuronal features of the AIY interneurons but is rather required for the subtype specification of this interneuron class. We describe several genes that participate in defining AIY cell fate and that represent either direct or indirect targets of the ttx-3 transcription factor. Neuroanatomical defects caused by loss of ttx-3 function resemble those seen in axon pathfinding mutants, suggesting that ttx-3 is also involved in determining patterns of axonal outgrowth. Furthermore, we show that a transcription factor regulated by ttx-3, the ceh-23 homeobox gene, participates in the regulation of a presumptive ttx-3 downstream target gene.

MATERIALS AND METHODS

Transgenic strains

Extrachromosomal arrays

adEx1450=Ex[ser-2::gfp; lin-15(+)] (courtesy of T. Niacaris and L. Averty)

otEx44 – otEx99, otEx97 – otEx101=Ex[txt-3full::gfp; pRF4]

otEx57=Ex[txt-3full::gfp; pBX]

otEx65=Ex[unc-119::txt-3; pRF4]

otEx75=Ex[unc-33::gfp; unc-4(+)]

otEx93=Ex[pflp-l1::gfp; pRF4]

otEx95, otEx96=Ex[unc-119::txt-3; unc-122::gfp]; unc-122::gfp is a new injection marker (see below)

utEx54=Ex[C36B7.7::gfp; pRF4] (courtesy of T. Ishihara and I. Katsura).

Chromosomally integrated arrays

evIs111=integrated Ex[F25B3.3::gfp; dpy-20(+)] (kindly provided by Joe Culotti)

kyIs5IV=integrated Ex[ceh-23::gfp; lin-15(+)] (Forrester et al., 1998)

mgs18IV and mgs32II were independently integrated Ex[txt-3prom::gfp; plin-15]

otIs7=integrated Ex[zig-2::gfp; pRF4] (O. Aurelio and O. H., unpublished)

otIs14=integrated Ex[zig-3::gfp; pRF4] (O. Aurelio and O. H., unpublished)

otIs24=integrated Ex[xre-1::gfp; dpy-20(+)]

otIs3IV=integrated Ex[kal-1::gfp; pBX] (H. Buelow and O. H., unpublished)

otIs45=integrated Ex[unc-119::gfp]

otIs62, otIs123=integrated Ex[psha-11.3::gfp; pRF4] and integrated Ex[psha-11.3::gfp; unc-4(+)], respectively

otIs97IV, otIs98, otIs99=integrated otEx65

otIs107=integrated adEx1450

otIs117=integrated otEx75.

The injection marker pBX was used in apha-1(e2123ts) background; unc-4(+), lin-15(+), and dpy-20(+) were used in unc-4(e120), lin-15(n765ts) and dpy-20(e1362) backgrounds, respectively; and pRF4 and unc-122::gfp in a wild-type background. (The protocol for integrating extrachromosomal arrays can be found at http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/hobert/protocols.htm)

Behavioral assays

Thermotaxis assays were performed as described (Hedgecock and Russell, 1975). In our specific set-up, an aluminum slab bridged two water baths, one at 61°C, the other at 5°C; the ambient temperature was 25°C. Animals were grown at 20°C and assayed on a gridded 10 cm square petri dish that had equilibrated on the aluminum plate to establish a stable gradient from 22°C to 16.5°C (measured with a surface temperature probe). Animals were placed into the center of the gradient. After an assay time of 40-60 minutes, the animals were anaesthetized. For scoring, the plate was divided into six regions of 1.3 cm width (see Fig. 3A) and animals were counted in each region. Animals that had not left the 1.7 cm² quadrant in which they had been initially placed were not scored.

Dauer assays were performed by allowing four gravid adults per plate to lay eggs for 20 hours at 15°C. Adults were picked off and their offspring cultivated on an OP50 seeded plate at 25°C. After 3.5 days, the brood was scored. Animals that showed the characteristic thin elongated shape of dauers and displayed a characteristic dauer cuticle were scored as dauers; all the remaining animals at a stage older than L3 were scored as non-dauers. Many of the daf-7; ttx-3 bypasser animals had transiently passed through a dauer-like, if not complete dauer stage, in which they remained for a variable amount of time. Hence, at the scoring time, dauer bypasser mutants were
observed at all different stages (L3 to adults). The ‘dauer bypass’ phenotype thus encompasses a failure to enter the dauer stage, as well as a failure to appropriately arrest in the dauer stage.

Isolation and analysis of ceh-23 null mutant animals
A ceh-23 null allele was isolated using a transposon-tagging strategy (Zwaal et al., 1993). First, a Tc1 insertion in the ceh-23 gene was isolated by PCR screening of a Tc1 library made from the MT3126 strain, using Tc1- and ceh-23-specific primers. The isolated strain YK16[mut-2(r459); ceh-23(ms16); Tc1]; dpy-19(n1347)III contained the Tc1 insertion in the last exon in the middle of the homeodomain at position 12957 (numbering of cosmid sequence ZK652). YK16 was then PCR screened for Tc1 excision events using ceh-23-specific primers. An imprecise Tc1 excision was identified in strain YK23[mut-2(r459); ceh-23(ms23)]; dpy-19(n1347)III at position 11098-12961. The junction sequence is: 5’-TTTAGGCTTGG[deletion]GTCGGAGACA-3’. YK23 was outcrossed several times with Bristol N2. As YK23 has no obvious phenotype, the genotype was scored using a triple PCR, in which the three primers U2, D2 and D3 gave a 1 kb product on homozygous ceh-23(23/23) animals (data not shown). Moreover, ADL

ceh-23::gfp expression in several neuron classes other than Aiy, including ADL, AWL, AFD, PHA, PHB and CAN (Forrester et al., 1998), prompted us to assay differentiation of some of these neurons. In ceh-23 null mutants. We found that the AFD and ASE cell fate markers gcy-5::gfp and ceh-23::gfp, respectively, the AFD, ADL, PHA, PHB cell fate marker srb-6::gfp, the AWC cell fate marker str-2::gfp and the CAN cell fate marker kal-1::gfp were all correctly expressed in ceh-23::gfp (data not shown). Moreover, ADL and PHA/PHB show normal dye filling behavior. In those cases where the neuron could be visualized in relative isolation (ADF, ASE, AWC, and PHA/PHB show normal dye filling behavior. In those cases where the neuron could be visualized in relative isolation (ADF, ASE, AWC, and PHA/PHB), the anatomy of the neuron appeared wild type.

Genetic screen for new ttx-3 alleles
To screen for new ttx-3 alleles, we made use of two previously described consequences of loss of ttx-3 function, the partial suppression of the dauer constitutive phenotype of a daf-7 loss-of-function allele and the loss of positive autoregulation of the ttx-3 gene (Hobert et al., 1997). A daf-7[e1372]III; mgsIs18IV strain, that contains an integrated ttx-3prom::gfp array, was mutagenized with EMS at the L4 larval stage. The F1 offspring were allowed to grow to adulthood at 25°C. Those plates were chunked and again grown to starvation at 25°C. Standard bleaching protocols. Approximately 100-300 eggs of this F2 generation were placed on single plates and cultivated at 25°C. Only plates that contained dauer bypass mutants quickly grew to starvation. Those plates were chunked and again grown to starvation at 25°C twice, in order to enrich for homozygous animals with the bypasser phenotype. Animals were isolated from plates that showed downregulated ttx-3prom::gfp expression, and after testing for X-linkage of the defect the ttx-3 genomic locus was sequenced. Out of >100,000 haploid genomes screened, we retrieved three ttx-3 alleles, mgs158, otEx95 and otEx96. In addition, we obtained another ttx-3 allele, nj14, that was isolated by Ikue Mori in an independent screen for mutants that affect ttx-3prom::gfp expression.

Pan-neuronal marker genes
We reasoned that the unc-33 gene, which appears to affect axon pathfinding throughout the nervous system (Hedgecock et al., 1985; McIntire et al., 1992), may be pan-neuronally expressed. We fused a 2.7kb genomic fragment that is immediately upstream to the start site of the previously described unc-33 mRNA transcript (Li et al., 1992) to gfp and found this reporter gene to be expressed throughout the whole nervous system (see Fig. 6A). Like unc-119::gfp (Maduro and Pilgrim, 1995), the unc-33::gfp reporter is expressed in dividing neuroblasts at pre-comma embryonic stages (data not shown). unc-33::gfp expression can be observed outside the nervous system in two amphid socket cells and weakly in non-neuronal pharyngeal cells. We also generated a fusion of the gfp gene to the promoter of the F25B3.3 gene, the C. elegans orthologue of the Ca2+-regulated ras nucleotide exchange factor CalDAG-GEFII/RasGRP, which is ubiquitously expressed in the vertebrate nervous system (Ebinu et al., 1998; Kawasaki et al., 1998). F25B3.3 and vertebrate CalDAG-GEFII/RasGRP both contain a unique combination of domains, a nucleotide exchange factor domain, two EF hands and a Cys-rich diacylglycerol binding motif; the respective C. elegans and rat proteins show 35% identity and 57% similarity throughout the whole length of the protein. The F25B3.3::gfp construct contains 3.5 kb genomic sequences upstream of the predicted ATG start site and the first six amino acids of the predicted protein and is exclusively expressed throughout the nervous system in C. elegans (see Fig. 6A). In contrast to unc-119::gfp and unc-33::gfp, which are already expressed in neuroblasts, F25B3.3::gfp is a postmitotic pan-neuronal marker, i.e. its onset of expression is observed after the terminal division of neurons (around 450 minutes of embryonic development; see Fig. 6B).

Pan-neuronal expression of ttx-3
The unc-119::ttx-3 construct contains the genomic coding region of ttx-3 from ATG to TGA cloned into the unc-119 expression vector pBY103 (Maduro and Pilgrim, 1995). This construct was injected at 10 ng/μl together with either prF4 (rol-6) or unc-122::gfp as injection marker into a mgsIs18; ttx-3(ks5) strain to yield three independent transgenic lines, otEx65, otEx95 and otEx96. All three lines show complete rescue of the autoregulatory defects of the ttx-3(ks5) mutation. otEx65 was chromosomally integrated to yield three independent integrants, otIs97, otIs98 and otIs99. All three extrachromosomal lines as well as all three integrated lines show ectopic ttx-3prom::gfp expression in RGD and CAN (CAN expression is less consistent in otEx95 and otEx96). otEx95 and otEx96 were assayed for thermotaxis behavior either in a wild-type or ttx-3(ks3) mutant background.

We examined ectopic expression of Aiy fate markers other than unc-33prom::gfp by using the extrachromosomal line otEx65, which we crossed with otIs33 (kar-1::gfp), kyls5 (ceh-23::gfp), otIs107 (ser-2::gfp) and otIs123 (sra-11::gfp). As kar-1::gfp and ser-2::gfp are already expressed in a substantial number of neurons in the head ganglia it was difficult to determine ectopic expression outside their normal expression domains in the head ganglia; it was clear, however, that pan-neuronal expression of ttx-3 did not cause a significant broadening of the expression of either of the markers in the nervous system.

UNC-17 antibody staining
We used a modification of the freeze-cracking method kindly provided to us by J. Duerr. Briefly, mixed stage animals that were grown at 20°C were transferred onto poly-L-lysine coated slides and covered with a coverslip. Slides were then fixed in liquid nitrogen and animals were cracked by flipping coverslips off. Animals were quickly fixed in methanol and acetone, incubated in blocking serum and co-stained with mouse monoclonal anti-UNC-17 (kindly provided by J. Duerr and J. Rand) and polyclonal anti-GFP (Molecular Probes) antibodies.

Other DNA constructs
The ttx-3 expression constructs ttx-3resc and ttx-3prom::gfp shown in Fig. 1B, have been described already (Hobert et al., 1997); ttx-5fida::gfp represents a GFP PCR fusion product in which the genomic region contained within ttx-3resc has been PCR-fused to gfp::unc-54-3 UTR from the pPD95.75 vector. (Protocol for PCR fusion can be found at: http://cpcnet.columbia.edu/dept/gsa/bioschem/labs/hobert/protocols.htm). The sra-11::gfp construct is termed prsa-11-3::gfp and contains
a 3.9 kb XbaI genomic fragment, which encompasses most of the coding region of sra-11 and 2.8 kb of sequence upstream of the predicted start codon. This construct is different from the one previously published (Troemel et al., 1995) and is more stably expressed in similar neuronal subtypes, namely AVB and AIV, but is also weakly expressed in AIA (see Fig. 5). The flp-1::gfp construct contains a genomic fragment from the 5' region of the flp-1 gene (Nelson et al., 1998), which extends from position −513 to −9 relative to the flp-1 start codon. This construct is strongly and exclusively expressed in the AVK interneuron pair. unc-122::gfp is a reporter gene construct that is strongly and exclusively expressed in coloemocytes and used as an injection marker (P. Loria and O. H., unpublished). Genomic fragments were amplified by PCR and subcloned into the standard GFP vectors pPD95.75 or pPD95.77. kal-1::gfp, zig-2::gfp, zig-3::gfp, ser-2::gfp and C36B7.7::gfp will be described elsewhere.

RESULTS

Expression of three homeobox genes in the AIY interneurons

We have previously shown that a genomic fragment that contains 3.1 kb of sequence 5' of the ttx-3 translational start site as well as the first two exons and introns of the ttx-3 gene directly expresses a gfp reporter gene to a single class of two bilaterally symmetric interneurons, AYIL and AYIR (Hobert et al., 1997). As additional regulatory elements for ttx-3 gene expression may exist in more downstream located introns, we inserted gfp coding sequences into a genomic construct, which we had previously shown to rescue the ttx-3 mutant phenotype; this construct contains 3.1 kb of 5' upstream sequence and all exons and introns of the ttx-3 gene (Fig. 1). Expression of this reporter gene construct is restricted to several head neurons throughout larval and adult stages (Fig. 1). The AIY interneuron class consistently expresses the reporter gene;
s slightly weaker but still very consistent expression is also observed in the AIA interneuron class and less consistent and weaker expression can be observed in the ADL and ASI sensory classes as well as two pharyngeal neurons.

As shown schematically in Fig. 1C, two other homeobox genes are also expressed in AIY, ceh-10 and ceh-23. ceh-10 was identified by sequence homology to homeobox genes (Hawkins and McGhee, 1990) and independently in a screen for mutants affecting neuronal migration (Forrester et al., 1998); it contains a paired-type homeobox and represents the C. elegans ortholog of the vertebrate ChaX10/ChaX10-1 genes, which are expressed in a restricted set of interneurons in the retina and spinal cord (Chen and Cepko, 2000). ceh-23 was identified due to its proximity to the C. elegans Hox cluster (Wang et al., 1993) and represents a diverged homeobox gene with no clear ortholog in other species and no conserved motif outside the homeodomain (Fig. 2A).

**Isolation of mutations in homeobox genes expressed in the AIY interneurons**

We sought to define the regulatory relationship of ceh-10, ttx-3 and ceh-23 as well as their function in AIY interneuron differentiation by analyzing the loss-of-function phenotypes of these genes. To this end, we used the previously identified ceh-10 null allele, gm58 (Forrester et al., 1998) and newly identified ttx-3 and ceh-23 loss-of-function alleles, which we describe below.

**ceh-23 mutant allele**

We isolated a ceh-23 deletion allele, ms23, through PCR-based screening of a Tc1 transposon library (see Materials and Methods). ceh-23/ms23 contains a 1864 bp deletion that extends from 301 bp 5’ of the ATG start codon into the last exon of the gene and deletes more than 75% of the coding sequence, including half of the homeodomain (Fig. 2B). It is thus likely that this allele represents a complete loss-of-function allele. Despite expression of ceh-23 in the CAN neurons, a neuron class that is essential for survival (Forrester et al., 1998).
Fig. 3. Phenotypic characterization of ttx-3 and ceh-23 alleles. (A) Thermotaxis assays (see Materials and Methods). Animals were grown at 20°C. Number of assays per genotype: wild type N2=11; ceh-23(ms23)=8; ttx-3(ks5)=6; ttx-3(mg158)=6; ttx-3(ot22)=7; ttx-3(ot23)=8. For each genotype, an average of 284-417 animals were tested per assay. The error bars represent the standard error of the mean. The difference in cryophilic behavior between wild type and ceh-23(ms23) is not statistically significantly different (P>0.3; paired and unpaired Student’s t-test). Our observation of a certain fraction of wild-type animals showing cryophilic behavior is consistent with previous reports (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). (B) Effect of ttx-3 alleles on the level of ttx-3prom::gfp expression in A1Y (white arrows point to A1Y/LR in different animals). All animals shown contain the integrated ttx-3prom::gfp array mgIs18, which was crossed into the respective mutant backgrounds. In ks5 mutant animals signal strength of mgIs18 is clearly reduced, yet enough freely diffusible GFP protein is made in the cell to allow visualization of the axons. In contrast, mgIs18 expression is mostly undetectable in mg158, ot22, and ot23 mutants. (C) Quantification of the defects shown in B. ‘Dim’ refers to clearly reduced gfp expression with the axons being barely visible, ‘very dim’ to gfp expression that is insufficient to visualize the axons. (D) Dauer assays (see Materials and Methods). Results are from four experiments.

Garriga, 1997), ceh-23(ms23) mutant animals are viable and display no obvious neuroanatomical, morphological or locomotory abnormalities (see Materials and Methods).

ttx-3 mutant alleles

Prior to this study, only one ttx-3 allele, ks5, was available. ks5 mutant animals show thermotaxis defects that are similar to those seen in animals in which the AIY interneurons have been ablated; additionally, when placed over a chromosomal deficiency, ttx-3(ks5) behaves as a complete loss-of-function allele (Hobert et al., 1997). However, the molecular nature of ks5, a splice site mutation, left open the possibility that small amounts of truncated and partially active TTX-3 protein could be generated through incorrect splicing. To undertake a detailed analysis of AIY interneuron fate in ttx-3 mutants, we thus sought to identify new mutant alleles of the ttx-3 gene, whose molecular nature may more unambiguously imply a complete loss of ttx-3 function. Through screening for mutants with characteristic ttx-3-like defects, we identified a total of three new ttx-3 alleles, termed mg158, ot22 and ot23 (see Materials and Methods). All three alleles result in characteristic thermotaxis defects (Fig. 3A), which mimic the thermotaxis defects seen upon laser ablation of the AIY interneurons (Mori and Ohshima, 1995). Each allele was

<table>
<thead>
<tr>
<th>ttx-3prom::gfp expression in A1Y</th>
<th>wildtype</th>
<th>ttx-3(ks5)</th>
<th>ttx-3(ot22)</th>
<th>ttx-3(ot23)</th>
<th>ttx-3(mg158)</th>
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<tr>
<td>strong</td>
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<td>10%</td>
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<td>0%</td>
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<td>75%</td>
<td>4%</td>
<td>2%</td>
<td>8%</td>
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<td>0%</td>
<td>0%</td>
<td>5%</td>
<td>2%</td>
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<tr>
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<td>0%</td>
<td>15%</td>
<td>75%</td>
<td>93%</td>
<td>90%</td>
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<td>n</td>
<td>94</td>
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<td>81</td>
<td>71</td>
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<table>
<thead>
<tr>
<th>genotype</th>
<th>bypasser at 25°C</th>
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</thead>
<tbody>
<tr>
<td>dpy-18(e1372); ttx-3(ks5)</td>
<td>1%</td>
<td>489</td>
</tr>
<tr>
<td>dpy-18(e1372); ttx-3(ot22)</td>
<td>63%</td>
<td>493</td>
</tr>
<tr>
<td>dpy-18(e1372); ttx-3(ot23)</td>
<td>53%</td>
<td>256</td>
</tr>
<tr>
<td>dpy-18(e1372); ttx-3(mg158)</td>
<td>82%</td>
<td>635</td>
</tr>
<tr>
<td>dpy-18(e1372); ceh-23(mg158)</td>
<td>1%</td>
<td>650</td>
</tr>
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are likely to be null for function (Fig. 2C). ttx-3 confirmed the notion that at least two of them (mg158, ot22) were identified based on their phenotype. Expression was never observed (n>50). (B) ceh-23::gfp expression is dependent on ttx-3. Adult ttx-3(mg158); kys5 were scored. Quantification is shown in Table 1. (C) ttx-3prom::gfp expression is unaffected in ceh-23(ms23) null mutants (n>50). Note the morphological integrity of the AIY neuron in terms of its cell position and axon morphology.

Table 1. Expression of AIY subtype markers in ttx-3 and ceh-23 mutant animals

<table>
<thead>
<tr>
<th>AIY cell fate marker*</th>
<th>Wild type†</th>
<th>ttx-3 (ks5)§</th>
<th>ttx-3 (mg158)§</th>
<th>ceh-23 (ms23)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1</td>
<td>Adult</td>
<td>L1</td>
<td>Adult</td>
</tr>
<tr>
<td>ttx-3</td>
<td>Homebox</td>
<td>100 (n=94)</td>
<td>99 (n=104)</td>
<td>7 (n=128)</td>
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<tr>
<td>ceh-23</td>
<td>Homebox</td>
<td>87 (n=55)</td>
<td>100 (n=67)</td>
<td>8 (n=40)</td>
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<td>Orphan 7TMR</td>
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<td>75 (n=49)</td>
<td>0± (n=75)</td>
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<tr>
<td>ser-2</td>
<td>Putative metabotropic 5-HT receptor</td>
<td>84 (n=32)</td>
<td>91 (n=24)</td>
<td>19 (n=21)</td>
</tr>
<tr>
<td>kal-1</td>
<td>Secreted protein</td>
<td>95 (n=21)</td>
<td>92 (n=92)</td>
<td>6 (n=29)</td>
</tr>
<tr>
<td>C36B7.7</td>
<td>Secreted protein</td>
<td>70 (n=36)</td>
<td>83 (n=143)</td>
<td>44 (n=54)</td>
</tr>
<tr>
<td>unc-17</td>
<td>Vesicular acetylcholine transporter</td>
<td>N.D.</td>
<td>87 (n=46)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

As residual expression of some AIY differentiation markers is still occasionally observed in the absence of ttx-3 function, we tested whether ceh-23 has a supplementary but non-essential role in ttx-3 dependent gene regulatory events. For example, a residual amount of CEH-23 protein may still be expressed in supplemen-tary but non-essential role in ceh-23. ttx-3 double mutants and be able to induce small amounts of expression of ttx-3-dependent target genes. We tested this possibility by examining AIY marker gene expression in ceh-23; ttx-3 double mutants and found that the defects were no worse than in the ttx-3 single mutant (data not shown). Thus, while ttx-3 and ceh-23 apparently both contribute to the regulation of the sra-11 gene, the regulation of other ttx-3 target genes appears to be entirely independent of ceh-23 function.

*Cell fate marker expression was monitored by antibody staining (unc-17) or by examining the expression of gfp reporter gene constructs (ttx-3: mg158, ceh-23: kys5, sra-11: ot22, ser-2: adEx1450, kal-1: ot33, C36B7.7: utEx54) that were crossed into the respective mutant backgrounds. Expression of the reporter gene in wild-type and mutant phenotypes were compared side-by-side.
†Numbers are percentages of animals that show expression of the respective marker in the AIY interneuron class.
§Numbers are percentages of mutant animals that show ‘wild-type-like’ expression levels of the respective marker. ‘Non-wild-type-like’ expression can be subdivided into weak but detectable expression or entirely absent expression.
¶We had previously noted effects of ttx-3(ks5) on sra-11::gfp expression from an extrachromosomal array, yet found a substantial number of ttx-3(ks5) animals that still express the reporter (Fig. 5B in Hobert et al., 1997). Here, we use a distinct sra-11::gfp construct (see Materials and Methods) that is expressed from a chromosomally integrated array.
N.D., not determined.

defective in autoregulation of a ttx-3prom::gfp reporter gene (Fig. 3B,C) and suppressed the dauer-constitutive phenotype of daf-7(e1372) at 25°C (Fig. 3D). While the behavioral defects of the new alleles were comparable with those seen in the previously available kss5 allele, the autoregulatory defects of the new alleles were stronger than those observed with the kss5 allele (Fig. 3B,C). Sequencing of the new ttx-3 alleles confirmed the notion that at least two of them (mg158, ot22) are likely to be null for ttx-3 function (Fig. 2C). ceh-10, ttx-3 and ceh-23 constitute a regulatory cascade of transcription factors in the AIY neurons

The availability of mutant alleles in all three homeobox genes has allowed us to address whether these transcriptional regulators act either in a linear pathway or, alternatively, in independent pathways within the AIY interneuron class. In a presumptive linear pathway, ceh-10 would likely be the most upstream acting gene, as ceh-10 expression in AIY is only observed during embryogenesis and fades after hatching.
These findings demonstrate that expression in AIY (Forrester et al., 1998) is required for sra-11::gfp expression was monitored by mating otIs62 animals that harbor an integrated sra-11::gfp construct with animals of the respective mutant backgrounds. The animals carry a rol-6 injection marker, leading to a slight distortion in the spatial arrangement of neurons. Quantification of the expression data in the AIY interneurons is shown in Table 1. While expression of sra-11::gfp in AVB is strong and highly penetrant in L1 larvae, it is observed less consistently and more weakly in adult animals (compare upper and lower panels; this observation was made with three independently created integrants, otIs62, otIs122 and otIs123). Expression of sra-11::gfp in AVB came to lie out of the plane of focus in the ceh-23(ms23); otIs62 adult animal shown in the lower right panel. Expression of sra-11::gfp in AIA is difficult to consistently detect in larvae, yet clearly visible in adult animals. 64% of wild-type adults show expression (n=45) in AIA and 38% (n=70) of ttx-3(mg158); otIs62 adults show expression in AIA (see lower middle panel).

Fig. 5. Expression of the AIY cell fate marker sra-11::gfp in ttx-3 and ceh-23 mutant animals. sra-11::gfp expression was monitored by mating otIs62 animals that harbor an integrated sra-11::gfp construct with animals of the respective mutant backgrounds. The animals carry a rol-6 injection marker, leading to a slight distortion in the spatial arrangement of neurons. Quantification of the expression data in the AIY interneurons is shown in Table 1. While expression of sra-11::gfp in AVB is strong and highly penetrant in L1 larvae, it is observed less consistently and more weakly in adult animals (compare upper and lower panels; this observation was made with three independently created integrants, otIs62, otIs122 and otIs123). Expression of sra-11::gfp in AVB came to lie out of the plane of focus in the ceh-23(ms23); otIs62 adult animal shown in the lower right panel. Expression of sra-11::gfp in AIA is difficult to consistently detect in larvae, yet clearly visible in adult animals. 64% of wild-type adults show expression (n=45) in AIA and 38% (n=70) of ttx-3(mg158); otIs62 adults show expression in AIA (see lower middle panel).

(AIY differentiation can be monitored by expression of a series of cell fate markers)

We next investigated how the ceh-10→ttx-3→ceh-23 regulatory cascade couples to AIY interneuron development and function. Cell type diversification in the nervous system is a consequence of differential gene expression within a given neuronal subtype. Hence, individual neuronal subtypes are defined by the expression of a specific and unique combination of molecular markers. The AIY interneuron class can be described by such a unique combinatorial gene expression profile: in a genome-sequence based survey of expression patterns of putative neuronal cell surface molecules, we identified a FnIII-domain-containing cell surface protein with homology to the human Kallmann’s syndrome gene, termed KAL-1, which is expressed in AIY and several other head neurons (H. Buelow, Z. A.-G. and O. H., unpublished). As the AIY neurons may constitute an integration point of temperature and food sensory inputs (Hobert et al., 1997; Mori and Ohshima, 1995) and since serotonin (5-HT) is involved in food signaling (Sze et al., 2000), we reasoned that a seven-transmembrane receptor with similarity to 5-HT and octopamine receptors, termed SER-2 (identified by T. Niacaris and L. Avery), is expressed in AIY. Moreover, an orphan serpentine receptor of unknown function, SRA-11 (Troemel et al., 1995), an acetylcholine transporter protein, UNC-17 (Alfonso et al., 1993; J. Duerr and J. Rand, personal communication) and a novel secreted protein of unknown function, C36B7.7 (T. Ishihara and I. Katsura, pers.com.) had been previously identified as being expressed in AIY and several other neurons. While the expression of none of these eight markers (ttx-3, ceh-23, kal-1, ser-2, sra-11, unc-17 and C36B7.7) is entirely restricted to AIY, the expression of all eight markers exclusively overlaps in the AIY interneuron class. AIY thus has a unique ‘address’, which defines its identity and allows it to be distinguished from other neurons. This ‘address’ has enabled us to investigate if and how the three homeobox genes ceh-10, ttx-3 and ceh-23 influence AIY interneuron differentiation.

ceh-23 is required to maintain one feature of the AIY differentiation program

We examined the function of ceh-23, the transcription factor that is most downstream in the ceh-10→ttx-3→ceh-23 cascade, by analyzing the expression of AIY marker genes in a ceh-23 null mutant background. We found that ceh-23 has no impact on the regulation of kal-1, C36B7.7, ser-2 and unc-17 expression, yet it affects expression of the orphan seven transmembrane receptor sra-11. While sra-11::gfp expression in ceh-23(ms23) L1 larvae appears normal, expression decreases significantly in adult animals (Fig. 5 and Table 1).
We also tested whether ceh-23 is required for the known functions of the AIY interneurons, thermotaxis and dauer arrest. Both behaviors were normal in ceh-23(ms23) mutant animals (Fig. 3A,D). We also observed that AIY interneuron structure, i.e. cell body position and axon morphology appears wild-type in ceh-23 null mutants (Fig. 4C). In summary, wild-type in ceh-23 animals (Fig. 3A,D). We also observed that AIY interneuron subtype-specific aspects of AIY differentiation characteristic, the expression of the sra-11 gene. Additionally, ceh-23 may affect other as yet unknown parameters of AIY structure and function.

**ttx-3 is required for the expression of all known subtype-specific aspects of AIY interneuron differentiation**

The finding that loss of ceh-23 function has neither an obvious effect on axonal morphology of the AIY interneurons nor on the known behaviors mediated by the AIY interneurons demonstrates that the function of ttx-3, the upstream regulator of ceh-23, must go beyond regulation of the ceh-23 gene, as ttx-3 animals display severe axonal and functional defects of the AIY interneurons (Fig. 3 and see Fig. 7). We examined parameters of AIY differentiation in ttx-3(mg158) mutant animals using the AIY cell fate markers described above. We found that the expression of every known AIY subtype marker depends on ttx-3 function (Fig. 5 and Table 1). Expression of ceh-23, kal-1, C36B7.7, ser-2, unc-17 and sra-11 is either significantly reduced or absent in ttx-3(mg158) and ttx-3(ks5) mutant animals (Fig. 5 and Table 1). As shown in Fig. 5, the effect of loss of ttx-3 function on expression of the sra-11 gene is stronger (loss in larvae and adults) when compared with the loss of ceh-23 function (loss only in adults), suggesting that the effect of ttx-3 on sra-11 gene expression cannot be solely explained by loss of ceh-23 function. Rather, both ttx-3 and ceh-23 seem to be contributing individually to the regulation of sra-11 expression. The finding that ttx-3 is required for expression of all known AIY subtype markers is in accordance with the complete loss of AIY function in thermotaxis and dauer arrest as shown in Fig. 3 and suggests that ttx-3 is a central regulator of AIY interneuron differentiation. At this point it can not be assessed how aberrant expression of these marker genes relates to the defects of AIY structure and function in ttx-3 mutants, as mutations in these marker genes have – with the exception of unc-17 – not been described so far. unc-17 null mutants die as embryos (Alfonso et al., 1993) and can not thus be readily tested for AIY structure and function. unc-17 hypomorphs show normal AIY morphology (see Table 3).

As loss of ttx-3 function causes the AIY interneurons to adopt a variably defective axon morphology which is unlike that of any other neuron, we consider it unlikely that a complete transformation from AIY fate to the fate of another neuron has taken place. We nevertheless tested this possibility by examining the expression of a spectrum of markers for other cell fates in ttx-3 mutant animals. We monitored fate markers expressed in cells that are related to AIY by either lineage, synaptic connectivity, function or axon morphology (Table 2). We found that none of these markers are ectopically activated in AIY in ttx-3(mg158) mutant animals and thus found no evidence that AIY has adopted another distinct cell fate features (Table 2). We instead favor the possibility that AIY has simply lost several, if not all, of its subtype-specific characteristics and remains in an at least partially undifferentiated state.

**ttx-3 and ceh-10 separate pan-neuronal from subtype-specific characteristics**

In ttx-3 mutant animals, the AIY interneuron class may have either completely lost all components of its neuronal identity or it may have exclusively lost subtype-specific aspects of its differentiation program, while still maintaining nonspecific aspects of neuronal identity. We set out to distinguish between these possibilities by examining the expression of reporter gene

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**Table 2. Neuronal cell fate analysis in ttx-3(mg158) animals**

<table>
<thead>
<tr>
<th>Neuronal cell type</th>
<th>Cell fate marker*</th>
<th>Expression in wild-type</th>
<th>Expression in ttx-3(mg158)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells that are related to AIY by lineage‡</td>
<td>SMDD</td>
<td>ZC21.2::gfp</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AVK</td>
<td>flp-1::gfp</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AWC</td>
<td>str-2::gfp</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AVL</td>
<td>unc-25::gfp</td>
<td>+</td>
</tr>
</tbody>
</table>

Cells that are synaptically connected to AIY and/or functionally related‡‡ | AIZ | lin-11::gfp | + | – |
| | AFD | gcy-8::gfp | + | – |
| | AWA | odr-10::gfp | + | – |
| | ASE | gcy-5::gfp | + | – |
| | AWC | str-2::gfp | + | – |

Cells with AIY-like axon projections and/or similar cell body position** | AIM | zyg-3::gfp | + | – |
| | AVK | flp-1::gfp | + | – |

Cells that express ttx-3 in wild-type animals | ADL | srb-6::gfp | + | – |
| | ADL | srb-7::gfp | + | – |
| | ASI | srd-1::gfp | + | – |
| | ASI | osm-10::gfp | + | – |
| | ASI | daf-7::gfp | + | – |
| | ASI | zig-2::gfp | + | – |
| | ASI | zig-3::gfp | + | – |
| | AIA | sra-11::gfp | +/+‡‡ | – |

*ZC21.2::gfp=kyIs123 (Colbert et al., 1997); flp-1::gfp=otEx93 (Materials and Methods); str-2::gfp=kyIs140 (Sagasti et al., 1999); unc-25::gfp=jaIs8 (Jin et al., 1999); lin-11::gfp=mglks21 (Hobert et al., 1998); gcy-8::gfp=nlIs1; gcy-8::gfp=alyIs17 (Yu et al., 1997); odr-10::gfp=kyIs37 (Sengupta et al., 1996); zig-2::gfp=alsIs7, zig-3::gfp=alsIs14 (O. Aurelio and O. H., unpublished); srb-6::gfp=miIs12; srb-7::gfp=miIs12; daf-7::gfp=saIs7 (Schackwitz et al., 1996). Reporter gene arrays were crossed into ttx-3(mg158) animals.

‡+, animals that show expression that is comparable with wild-type expression (n=20).

‡‡This category includes neurons that show a lineage history that differs from AYL/R at only one step in the invariant cell division pattern that generates AYL/R (Sulston, 1983).

‡‡‡AIY receives major synaptic input from the AFD, AWA, AWC and ASE sensory neurons and makes prominent synapses onto AIZ (White et al., 1986). AFD, AIY and AIZ are functionally interconnected neurons in the thermotaxis circuit (Mori and Ohshima, 1995).

**AIML, AYL and AVKL are located in a characteristic row of three cells behind the excretory cell in adult animals (Sulston et al., 1983; White et al., 1986). Additionally, AIM extends a monopolar axon into the nerve ring that closely resembles that of AYL (White et al., 1986).

‡‡‡‡sra-11::gfp expression of the otIs62 strain in the AIA interneurons is observed in 64% of wild-type animals (n=45) and 38% (n=70) of ttx-3(mg158); otIs62 animals.

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[49x393]ttx-3 is required for the expression of all known subtype-specific aspects of AIY interneuron differentiation

[15x470]The finding that loss of ceh-23 function has neither an obvious effect on axonal morphology of the AIY interneurons nor on the known behaviors mediated by the AIY interneurons demonstrates that the function of ttx-3, the upstream regulator of ceh-23, must go beyond regulation of the ceh-23 gene, as ttx-3 animals display severe axonal and functional defects of the AIY interneurons (Fig. 3 and see Fig. 7). We examined parameters of AIY differentiation in ttx-3(mg158) mutant animals using the AIY cell fate markers described above. We found that the expression of every known AIY subtype marker depends on ttx-3 function (Fig. 5 and Table 1). Expression of ceh-23, kal-1, C36B7.7, ser-2, unc-17 and sra-11 is either significantly reduced or absent in ttx-3(mg158) and ttx-3(ks5) mutant animals (Fig. 5 and Table 1). As shown in Fig. 5, the effect of loss of ttx-3 function on expression of the sra-11 gene is stronger (loss in larvae and adults) when compared with the loss of ceh-23 function (loss only in adults), suggesting that the effect of ttx-3 on sra-11 gene expression cannot be solely explained by loss of ceh-23 function. Rather, both ttx-3 and ceh-23 seem to be contributing individually to the regulation of sra-11 expression. The finding that ttx-3 is required for expression of all known AIY subtype markers is in accordance with the complete loss of AIY function in thermotaxis and dauer arrest as shown in Fig. 3 and suggests that ttx-3 is a central regulator of AIY interneuron differentiation. At this point it can not be assessed how aberrant expression of these marker genes relates to the defects of AIY structure and function in ttx-3 mutants, as mutations in these marker genes have – with the exception of unc-17 – not been described so far. unc-17 null mutants die as embryos (Alfonso et al., 1993) and can not thus be readily tested for AIY structure and function. unc-17 hypomorphs show normal AIY morphology (see Table 3).

As loss of ttx-3 function causes the AIY interneurons to adopt a variably defective axon morphology which is unlike that of any other neuron, we consider it unlikely that a complete transformation from AIY fate to the fate of another neuron has taken place. We nevertheless tested this possibility by examining the expression of a spectrum of markers for other cell fates in ttx-3 mutant animals. We monitored fate markers expressed in cells that are related to AIY by either lineage, synaptic connectivity, function or axon morphology (Table 2). We found that none of these markers are ectopically activated in AIY in ttx-3(mg158) mutant animals and thus found no evidence that AIY has adopted other distinct cell fate features (Table 2). We instead favor the possibility that AIY has simply lost several, if not all, of its subtype-specific characteristics and remains in an at least partially undifferentiated state.

**ttx-3 and ceh-10 separate pan-neuronal from subtype-specific characteristics**

In ttx-3 mutant animals, the AIY interneuron class may have either completely lost all components of its neuronal identity or it may have exclusively lost subtype-specific aspects of its differentiation program, while still maintaining nonspecific aspects of neuronal identity. We set out to distinguish between these possibilities by examining the expression of reporter gene
tools that monitor aspects of pan-neuronal and non-subtype-specific neuronal differentiation. Besides the previously described *unc-119* gene (Maduro and Pilgrim, 1995), we constructed two other pan-neuronally expressed marker genes, *unc-33::gfp* and *F25B3.3::gfp* (see Materials and Methods). *unc-33::gfp*, like *unc-119::gfp*, marks all neuroblasts and differentiated neurons, while *F25B3.3::gfp* is a marker for all postmitotic neurons (Fig. 6A and Materials and Methods). We crossed each of the three pan-neuronal marker genes into *ttx-3(mg158)* mutant animals and found that loss of *ttx-3* function has no appreciable effect on the expression of any of these marker genes in AIY (Fig. 6C). These findings show that the adoption of subtype-specific characteristics can be separated from the adoption of pan-neuronal characteristics and that *ttx-3* is required for the former but not the latter aspect of AIY development.

As *ceh-10* acts upstream of *ttx-3*, it can be assumed that AIY development is at least as severely affected in *ceh-10* mutants as it is in *ttx-3* mutants. Consistent with this notion we found that several AIY subtype markers failed to be expressed in *ceh-10-null* mutants (Fig. 4 and data not shown). To address whether AIY lost pan-neuronal characteristics in *ceh-10-null* mutants, we examined expression of the *F25B3.3::gfp* pan-neuronal marker in *ceh-10-null* mutants and found it to be unaffected (Fig. 6C). The integrity of AIY generation and positioning in *ceh-10(gm58)* had also been noted previously by DIC microscopy (Forrester et al., 1998). Thus, *ceh-10* and *ttx-3* both act downstream or in parallel to the determination of the subtype-specific differentiation program.

**Ectopic *ttx-3* expression reveals *ceh-10*-dependent constraints on *ttx-3* function**

The results shown above reveal that *ttx-3* is an essential regulator of subtype-specific aspects of the AIY differentiation program. To address whether *ttx-3* is also sufficient to induce AIY-like features in other neurons, we ectopically expressed *ttx-3* throughout the nervous system, using the pan-neuronal
unc-119 promoter (see Materials and Methods). Several independently obtained transgenic ttx-3(ks5) mutant animals that express unc-119::ttx-3 show normal expression of ttx-3prom::gfp in the AIY interneurons and are thus rescued for the autoregulatory defect of ttx-3 expression (Fig. 7). All transgenic lines are viable and show no obvious morphological abnormalities (see Materials and Methods). A pan-neuronally expressed gfp reporter construct that visualizes several structural aspects of the nervous system (including presence and localization of axon fascicles; fasciculation within nerve bundles; presence and localization of ganglia) reveals no obvious abnormalities in animals that express ttx-3 pan-neuronally (data not shown). Intriguingly, unc-119::ttx-3 transgenic lines behave indistinguishably from wild type in thermotaxis assays, either in the presence or absence of endogenous ttx-3 gene activity (data not shown); thus, unc-119::ttx-3 can substitute for loss of endogenous ttx-3 function but is not sufficient to functionally disrupt other neurons in the thermotaxis circuit.

We tested whether other neurons adopted AIY-like characteristics by examining the expression of five AIY cell fate markers, ttx-3prom::gfp, ceh-23::gfp, sra-11::gfp, kal-1::gfp and ser-2::gfp in transgenic animals that express unc-119::ttx-3 (see Materials and Methods). Pan-neuronal expression of ttx-3 did not lead to a widespread expansion of the expression domains of any of the five markers. However, ectopic expression of the ttx-3prom::gfp fate marker could be unambiguously observed in two classes of neurons, the RID motoneuron and the CAN neurons (Fig. 7A). Ectopic expression of the ceh-23::gfp marker as well as the sra-11::gfp marker could also be detected in RID (Fig. 7B). Ectopic expression of the ser-2::gfp and kal-1::gfp marker could not be tested, as both markers are already expressed in RID in wild-type animals. Similarly, ceh-23::gfp, ser-2::gfp and kal-1::gfp are already expressed in CAN in wild-type animals. Hence, RID and CAN already share several characteristics with AIY in wild-type animals, yet ttx-3 is able to confer even more AIY-like characteristics to these cells upon ectopic expression (Fig. 7D).

Fig. 7. Effects of ectopic ttx-3 expression and dependence on ceh-10 activity. (A,B) RID and CAN can adopt AIY-like features upon ectopic expression of ttx-3. (A) A transgenic animal is shown that expresses ttx-3prom::gfp (normally only expressed in AIY; see Fig. 3B) and unc-119::ttx-3 from the independently integrated arrays mgIs18 and otIs97IV, respectively, in a ttx-3(ks5) background. Note that the autoregulatory defect of ttx-3prom::gfp in AIY is completely rescued and that ectopic expression is induced in RID and CAN. The animal is slightly twisted owing to the presence of a rol-6 injection marker. Ectopic RID and CAN expression could be observed with several independent transgenic lines (see Materials and Methods). (B) Ectopic expression of ceh-23::gfp in RID upon pan-neuronal ttx-3 expression. The genotype of this strain is kyls5IV; otEx65. Eleven out of 14 examined adults show expression in RID. kyls5 animals never show GFP expression in RID (n>20). (C) ceh-10 dependence of ectopic ttx-3prom::gfp expression. Homozygous ceh-10(gm58) offspring of ceh-10(gm58)/+; otIs97mgIs18IV hermaphrodites arrest as L1 larvae and were identified based on their clr phenotype. In these animals, no GFP signal can be detected in RID, CAN, or AIY (the approximate position of the AIY interneurons is indicated with an arrow), showing that expression of ttx-3 from an exogenous promoter can not rescue the ceh-10(gm58) phenotype. White dots derive from autofluorescent gut granules. (D) Summary of expression of several AIY cell fate markers in RID and CAN either in wild-type animals or in animals that pan-neuronally express ttx-3. Ectopic sra-11::gfp expression in RID was observed in 19/19 animals (data not shown) and was never observed in wild-type animals (n>20).
Fig. 8. Characterization of the neuroanatomical defects in ttx-3 mutant animals. (A) Axonal defects observed in various mutants. The wild-type AIY axon is shown in black. In wild-type animals, a short process (shown in dark gray) can occasionally be observed in the region where the main axon turns dorsally to enter the nerve ring; those are not scored as a mutant phenotype, unless they are longer than 10 μm (shown as light gray addition to the dark gray line). Schematic examples of aberrant axon sprouts are in light gray. We set the minimum length for scoring aberrant sprouts that emanate from the axon arbitrarily at 2 μm. As cell bodies occasionally narrow into a thin ending, we arbitrarily set the criteria to score sprouts from the cell body more stringently at 4 μm. Occasional ‘splits’ of the axon in the nerve ring region are counted as sprouts. Small enlargements along the axon, rarely observed in wild-type animals, often observed in pathfinding mutants, are not scored as sprouts. Every case in which the main axon does not meet the axon of its contralateral homolog at the dorsal midline is classified as ‘short stop’; the main axon stalls either at the turning point into the nerve ring (but never before that point), or at various points below or above the dorsoventral midline (black arrows). (B) Characteristic examples of axonal defects observed in ttx-3 mutants. AIY is visualized with mgIs18. Quantitative data are shown in Table 3. Similar results have also been obtained with several extrachromosomal ttx-3prom::gfp lines (Hobert et al., 1997). For this analysis we could make use only of the ttx-3(ks5) allele because, owing to the more severe reduction of ttx-3prom::gfp expression in the stronger ttx-3 alleles, the axon of the AIY interneurons cannot be visualized. It is thus possible that in the stronger alleles AIY neuroanatomy is even more strongly affected. (C) Representative examples of AIY neuroanatomy in unc-33(e120), unc-73(e936) and unc-119(e2498) mutant animals, visualized with mgIs18. White triangles point to short stops, white arrows to sprouts. Animals shown in this figure are late larvae and young adults. Notably, besides the short-stop defect of the main axon of the AIY neurons, these axon pathfinding mutants also cause axon sprouting defects. As neural activity defects do not cause axon sprouting in AIY (Table 3), the axon sprouting observed in pathfinding mutants are unlikely to be a secondary consequence of aberrant connectivity caused by aberrant pathfinding. We rather hypothesize that the axon sprouting defect observed in pathfinding mutants as well as in ttx-3 mutants reflects an inability of the neuron to initiate and maintain the proper outgrowth of a single, monopolar axon.

Notably, the only two neuron classes in which AIY-like characteristics could be induced by pan-neuronal ttx-3 expression, RID and CAN, normally express ceh-10 (Fig. 1), suggesting that it is the presence of ceh-10 that constrains ectopic ttx-3 activity to these neurons. We tested this hypothesis by examining ectopic ttx-3prom::gfp expression in RID and CAN in animals that carry an integrated unc-119::ttx-3 expressing array, but lack ceh-10 function. We found that in these animals ttx-3prom::gfp expression is downregulated or completely turned off in RID, CAN and AIY (Fig. 7C). This observation points to a requirement of ceh-10 for ttx-3-dependent regulatory events. The mere co-existence of ceh-10 and ttx-3 is however not sufficient to confer AIY-like characteristics to a neuron, as pan-neuronal misexpression of ttx-3 does not appear to induce the expression of AIY-like characteristics in other ceh-10-expressing cells (such as RMED, AIN etc.; data not shown). We conclude that ttx-3 is able to regulate the expression from its own promoter and of presumptive target gene promoters only in particular cellular contexts, which are directly or indirectly dependent on the presence of the ceh-10 homeobox gene.

**ttx-3 is also implicated in axon outgrowth and pathfinding**

Our identification of genes whose expression depends on ttx-3 function represents one route to assess the impact of ttx-3 on AIY differentiation. Another approach is to find genes whose mutant phenotype is similar to the ttx-3 mutant phenotype and whose function may hence be related to ttx-3 function. We have previously shown that the AIY interneurons display a characteristic set of neuroanatomical defects in ttx-3 mutant animals. These defects included inappropriate termination of the main axon and sprouting of additional neurites (Hobert et al., 1997; see also Fig. 8). Recently, ectopic neurite sprouting in *C. elegans* was described as a secondary consequence of defects in neuronal activity of sensory neurons and
Avery neuroanatomy was scored in adult animals using the integrated ttx-3::gfp array mgIs18 if not indicated otherwise; the scoring criteria are detailed in Fig. 8A. Percentages refer to the number of animals in a population with a given defect; defects of >10% penetrance are shaded. References for mutants can be found elsewhere (Antebi et al., 1997; Culotti, 1994; Rand and Nonet, 1997).

Table 3. Axon morphology of the AIY interneuron class in defined mutant backgrounds

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Adult</th>
<th>Animals with short stop (%)</th>
<th>Animals with sprouts (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (mgIs18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>ttx-3(ks5)*</td>
<td>L1</td>
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<td>2</td>
<td>104</td>
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<td>L2</td>
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<td>L4</td>
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<td>Adult</td>
<td>22</td>
<td>55</td>
<td>76</td>
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Sensory mutants:

daf-6(e1377), che-3(e1224), che-11(e1810), osm-6(p811), tax-2(p691), tax-4(p678)

<2 0 >45

Synaptic transmission mutants:

unc-64(trc246), unc-18(e81), unc-13(e51,e1091,s69), aex-3(n2166), raf-3(js49), unc-31(e928), unc-104(eb43), snb-1(e1254), js124, flp-1(yn2), unc-17(e245), unc-7(e5)

<3 1 >50§

Membrane potential and Ca2+ signaling

unc-36(e251), unc-2(mu74), egl-36(n728), egl-19(ad695gf), egl-19(n2368gf), eat-6(ad467)

<3 <2 >48

Axon outgrowth and pathfinding mutants:

Intracellular

unc-33(e204) (CRMP) 53 25 80

unc-44(e362) (ankyrin) 61 61 74

unc-51(e369) (Ser/Thr kinase) 11 11 115

unc-53(e404) 32 3 119

unc-73(e926) (rho/ rac GEF) 70 17 78

unc-76(e911) (FEZ1) 97 33 103

unc-115(mn481) (LIM domains) 3 2 67

unc-119(e2498) 100 46 63

Transmembrane/secreted

sax-3(ky123) (Robo) 90 49 91

vat-1(d31) (Eph-R) 0 6 66

unc-40(e1430) (Netrin-R) 7 14 81

unc-35(e53) (Netrin-R) 1 7 114

unc-6(e400) (Netrin) 23 12 71

ina-1(e1144) (α-Integrin) 92 16 98

Others

unc-43 (n498gf) (CamKII) 0 0 47

unc-43(e409f) 0 0 88

sax-2(a10) 0 55 67

lin-12(e941) (Notch) 3 (0%) 4 (5%) 103 (41%)

*This category includes mutants shown to cause axon sprouting in other contexts, such as sax-2, which causes sensory axon sprouting in C. elegans (Zallen et al., 1999), CamKII, which regulates axon sprouting in C. elegans and in the frog optic tectum (Wu and Cline, 1998; Zallen et al., 1999) and Notch, which regulates sprouting in the cerebellar cortex (Sestan et al., 1999) and axon pathfinding in the fly CNS (Giniger et al., 1993). sax-2(ot10)/III is a newly identified allele of the previously described sax-2 gene (Z. A.-G. and O. H., unpublished).

†††This result differs from a recent report that showed AY axon morphology defects in lin-12(e941) mutants (Wittenburg et al., 2000). We examined AY anatomy in the lin-12(e941) null mutant using either of two independently generated ttx-3::gfp integrants, mgls18 or mgls32 to exclude strain background artifacts. The unc-13(e51) allele (strain name: RM2423) is a four times back-crossed derivative of the canonical unc-13(e51) allele (strain name: RM2423). 18 animals were scored for the snb-1 null allele js124 (a gift from Mike Nonet), which die as L1s.

¶Most of the alleles shown in this category are null alleles.

†‡‡This result differs from a recent report that showed AY axon morphology defects in lin-12(e941) mutants (Wittenburg et al., 2000). We examined AY morphology in the lin-12(e941) null mutant using either of two independently generated ttx-3::gfp integrants, mgls18 or mgls32 (numbers shown in parenthesis). lin-12(e941); mgls18 and lin-12(e941)mgls32 were each balanced with the lin-12 rescuing array arEx29 (Fitzgerald et al., 1993). AY anatomy was scored in animals that had lost the balancing arEx29 array, and hence were non-rolling and had a large blip at the vulva. Similar results were obtained when lin-12(e941) was balanced with a lin-12(+)/chromosome (data not shown). We never observed any of the severe axonal defects reported in any of these strains (Wittenburg et al., 2000). In contrast to Wittenburg et al., we also did not observe AY axonal defect in sel-12/presenilin mutants (using a newly isolated null allele of the sel-12 presenilin gene; I. Temkin and I. Greenwald, personal communication) or in egl-19(n2368gf) mutants; additionally, no defects could be observed in another egl-19 gain-of-function allele or in any other synaptic transmission/electrical activity mutant shown in the Table. As we observed axonal defects of AY in several defined genetic backgrounds (as shown in the Table; H. Buelow, K. Berry and O. H., unpublished) with either of two independently integrated reporter gene arrays, as well as with extrachromosomal arrays, the presence of any linked suppressor mutations in our strain backgrounds can be excluded. As Wittenburg et al. used distinct ttx-3::gfp extrachromosomal arrays for the observation of AY defects and for their rescue experiments, it cannot be excluded that the effects that they observe are due to a partial defectiveness differentially induced by distinct ttx-3::gfp arrays (notably, the morphology of AY looks irregularly shaped in a wild-type animal that expresses a ttx-3::gfp array; Wittenburg et al., 2000) and/or due to mutations in their strain background.
motoneurons (Peckol et al., 1999; Zhao and Nonet, 2000). In contrast to their capacity to induce sprouting in motoneurons (Zhao and Nonet, 2000), we found that the synaptic transmission mutants unc-18(e81) and unc-13(e51), and the neurotransmitter transport mutant unc-17(e245) have no effect on AIY axon morphology (Table 3). Similarly, several other tested neurotransmission mutants also do not cause any neuroanatomical defects in AIY (Table 3). We additionally tested the outcome of electrical silencing of AIY by individually expressing two gain-of-function K+ channel mutant subunits, EGL-36 (E142K, P489S) (Johnstone et al., 1997) and EGL-2 (A478V) (Weinshenker et al., 1999) under the control of an AIY-specific ttx-3 promoter fragment and found no effect on AIY neuroanatomy (data not shown).

Furthermore, sensory activity mutants that cause neurite sprouting in sensory neurons, such as tax-2, tax-4, eat-6, che-3 and others (Peckol et al., 1999) have no effect on AIY axon morphology (Table 3). Additionally, while neuronal-activity dependent axonal sprouting of sensory neurons and motoneurons has recently been shown to be strongly influenced by ambient temperature and the developmental stage of the animal (Zhao and Nonet, 2000; Peckol et al., 1999; P. Loria and O. H., unpublished), we found no impact of these parameters on the ttx-3(ks5) induced sprouts (Table 3; data not shown).

Taken together, unlike the situation in defined sensory and motoneurons, the absence of synaptic transmission is unlikely to account for the neurite sprouting defects in ttx-3 mutant animals. We favor the possibility that the axonal defects observed in ttx-3 mutant animals represent developmental defects during the phase of axonal outgrowth. To test this notion, we compared the qualitative appearance and penetration of axonal sprouts observed in several axon pathfinding mutants with those seen in ttx-3(ks5) mutants (ks5 is the only allele that allows visualization of AIY neuroanatomy; Fig. 3). We found that mutations in all almost all of the intracellularly acting outgrowth/pathfinding proteins, which are thought to act throughout the nervous system (Antebi et al., 1997; Culotti, 1994; Hedgecock et al., 1985; McIntire et al., 1992), show defects that resemble those seen in ttx-3 mutants (Fig. 8; Table 3). Furthermore, we found that mutations in the cell-specific axon guidance cues unc-6/Ntrn (Hedgecock et al., 1990), sax-3/Robo (Zallen et al., 1998) and ina-1/Integrin (Baum and Garriga, 1997), but not in vab-1, the only ephrin receptor in C. elegans (George et al., 1998), or in lin-12/Notch (Giniger et al., 1993; Greenwald et al., 1983) cause axonal defects in the AIY interneurons (Table 3). This is an intriguing finding, as unc-6, sax-3 and ina-1 have not been previously implicated in axon outgrowth into the nerve ring. unc-6 null mutants show normal guidance of amphid sensory axons in the nerve ring (Hedgecock et al., 1990), ina-1 hypomorphic mutants show fasciculation defects, but no apparent outgrowth defects of sensory axons in the nerve ring (Baum and Garriga, 1997) and sax-3 null mutants were reported to show displacements of the nerve ring, but only subtle and cell type-specific premature termination of nerve ring axons (Zallen et al., 1999). The impact of unc-6, sax-3 and ina-1 on AIY axon outgrowth in the nerve ring is thus unlikely, owing to the disruption of nerve ring pioneer neurons because that would presumably cause more pleiotropic axon outgrowth defects in the nerve ring. The netrin, robo and integrin guidance systems may act directly in the AIY interneuron class. Whether components of these guidance systems are under direct control of the TTX-3 homeodomain protein remains to be investigated.

Given the similarity in phenotypes observed between axon outgrowth/pathfinding mutants and ttx-3 mutants, we hypothesize that ttx-3 is required to regulate correct axon outgrowth and pathfinding of the AIY interneurons. As ttx-3 appears to have no effect on the expression of pan-neuronal characteristics (Fig. 6), we hypothesize that cell-type specific aspects of AIY axon outgrowth, such as the expression of a guidance receptor, rather than pan-neuronal aspects of axogenesis, are affected in ttx-3 mutants.

**Role of ttx-3 in the differentiation of other neuron classes**

In contrast to its pronounced effect on AIY interneuron differentiation, ttx-3 does not appear to influence the adoption of several subtype-specific differentiation features of the ttx-3-expressing sensory neuron classes ADL and ASI. The expression of five differentiation markers of the ASI sensory neurons (daf-7::gfp, osm-10::gfp, sre-1::gfp, zig-2::gfp and zig-3::gfp) was unaffected in ttx-3(mu158) mutants (Table 2). Similarly, the ADL sensory neurons still expressed the putative odorant receptors srb-6 and sre-1 in the absence of ttx-3 activity (Table 2). Both ADL and ASI are also still capable of being filled with the vital dye DiI in ttx-3 mutant animals, suggesting that these sensory neurons do not require ttx-3 to assemble intact, exposed sensory endings.

We examined the fate of the AIA interneuron class in ttx-3 mutants and found that expression of the only two known marker genes of AIA fate are partially affected. srx-11::gfp expression from the otsl62 array can be observed in 64% of ttx-3(+) animals (n=45) and 38% (n=70) of ttx-3(mu158) animals (Fig. 5); UNC-17 antibody staining in AIA can be observed in 94% of wild-type animals (n=32) and 66% (n=58) of ttx-3(ks5) mutant animals (a ttx-3(ks5); mgIs18 strain was used, which in contrast to the stronger ttx-3 alleles permits an unambiguous identification of the AIA neurons by co-staining with anti-GFP antibodies). Additionally, the AIA interneurons appear to display neuroanatomical defects (data not shown). Further cell fate markers and functional assays for the AIA interneurons need to be established in order to fully assess the impact of ttx-3 on AIA differentiation and function.

**Regulatory relationship between ceh-10, ttx-3 and ceh-23 in other neurons**

Based on reporter gene analysis, the AIY interneuron class appears to be the only neuron class in which the expression of the three homeobox genes ceh-10, ttx-3 and ceh-23 overlaps. The otherwise largely non-overlapping expression pattern of these genes illustrates that ceh-10 is neither sufficient to turn on ttx-3 expression in other neuron classes (RMED, RID, CEP, ALA, AVJ, AIN or CAN) nor necessary to turn on ttx-3 expression in the ADL, ASI or AIA neuron classes. In addition, ttx-3 is neither sufficient to turn on ceh-23 expression in ASI or AIA nor necessary to turn on ceh-23 expression in several other sensory neuron classes (Fig. 1C). However, there are two neuron classes in which the expression of two of the three homeobox genes overlaps (ADL, ttx-3 and ceh-23; CAN, ceh-10 and ceh-23), thus raising the question whether the regulatory relationship between these genes, observed in the AIY interneuron, also exists in ADL and CAN. We found that...
ceh-23 expression in ADL was unaffected in ttx-3(mg158) mutant animals (data not shown). Thus, the regulatory relation between ttx-3 and ceh-23 is not conserved in neurons other than the AIY interneuron class. In contrast, in the CAN neurons, in which ceh-10 expression overlaps with ceh-23 expression, ceh-10 regulates ceh-23 expression (Forrester et al., 1998). However, this regulation is independent of ttx-3 which is not expressed in CAN. Accordingly, ceh-23 expression in CAN is unaffected in ttx-3(mg158) mutant animals (data not shown). Thus, a regulatory relationship between these three homeobox genes exists in one neuron class (AIY), partially in another neuron class (CAN) and not at all in another neuron class (ADL).

**DISCUSSION**

We have described here a regulatory cascade of three transcription factors which are involved in controlling AIY interneuron differentiation (Fig. 9). All three transcription factors are expressed and appear to function after the terminal cell division that generates the AIY interneuron class. ceh-10 and ttx-3 determine all known aspects of subtype-specific AIY interneuron fate while ceh-23 ensures maintained expression of one AIY differentiation feature (Fig. 9). While transcriptional regulatory cascades have previously been described in several developmental contexts in the nervous system (Anderson and Jan, 1997; Jurata et al., 2000), they have only rarely been identified in postmitotic neurons and the absence of appropriate cell fate markers has usually precluded an in-depth analysis of the role of transcription factor cascades in the differentiation of defined neuron classes. It is presumably the complexity of these postmitotically acting transcriptional networks that translates cell lineage history and environmental cues into the generation of the enormous variety of cell types in the nervous system.

**Role of ceh-10 in AIY development**

As ceh-10 regulates ttx-3, which in turns regulates all known aspects of AIY development and function, it is not immediately apparent what distinguishes the function of ceh-10 and ttx-3. As ceh-10 is only expressed embryonically, whereas ttx-3 and all its target genes are expressed embryonically and post-embryonically, we consider it unlikely that ceh-10 and ttx-3 act strictly together as co-factors on both the ttx-3 promoter and then, subsequently, the target gene promoters. However, it can not be the only role of ceh-10 to initiate ttx-3 gene expression that then serves to regulate its own expression, as expression of ttx-3 under control of the pan-neuronal, and presumably non-ceh-10-dependent unc-119 promoter is not able to induce expression from the ttx-3 promoter in the AIY interneurons in ceh-10 null mutant animals. Also, ectopic ttx-3 is not sufficient to induce expression from its own promoter in non-ceh-10-expressing neurons. ceh-10 thus appears to provide means to allow the TTX-3 protein to regulate its own expression; one out of several possible scenarios is that ceh-10 initiates the expression of yet another transcription factor(s) that acts together with TTX-3 to regulate expression from the ttx-3 promoter and possibly other target gene promoters. In addition to its role in regulating ttx-3 expression, ceh-10 could be involved in the regulation of other as yet unknown and ttx-3-independent AIY differentiation characteristics.

**ttx-3 is a key regulator of AIY interneuron differentiation**

The understanding of the function of Lhx genes in neuronal development has in many instances been hampered by a lack of molecular markers, which precluded the precise assessment
of the consequences of absence of Lhx gene function. By establishing a set of AIY differentiation markers and by visualizing axon morphology using a single neuron class-specific marker, we have carried out a detailed characterization of the consequences of loss of \textit{ttx-3} function. In \textit{ttx-3} mutants, expression of all known AIY cell fate markers is severely affected; moreover, the neuroanatomical defects of AIY in \textit{ttx-3} mutants resemble the defects seen in axonal pathfinding mutants. We conclude that \textit{ttx-3} is required to determine most, if not all subtype-specific aspects of the developmental characteristics. This can be inferred from the neuron-like neuronal features, TTX-3 does not determine pan-neuronal in vitro DNA binding assays.

In spite of its prominent role in determining subtype-specific neuronal features, TTX-3 does not determine pan-neuronal characteristics. This can be inferred from the neuron-like appearance of AIY in \textit{ttx-3} mutant animals (e.g. the AIY cell body extends an axon projection towards the nerve ring) and from the observation that the expression of three pan-neuronal markers is unaffected in \textit{ttx-3} mutants. These findings show that adoption of pan-neuronal features is separable from the adoption of more cell-type restricted features, and show that \textit{ttx-3} genetically separates these two aspects of neuronal differentiation.

Although \textit{ttx-3} is required in AIY to adopt all of its known cell-type specific characteristics, \textit{ttx-3} is not sufficient to impose a complete AIY differentiation program onto other neurons. Although ectopic \textit{ttx-3} expression can induce the adoption of several AIY-like characteristics, it can only do so in two neuron classes (RID and CAN) and even in those cases \textit{ttx-3} is not capable of conferring AIY-like structural features (cell position, axonal anatomy) onto these neurons. It is presumably the absence of an appropriate transcription factor ‘code’ (discussed below) that does not allow TTX-3 to confer AIY-like features to more than two other neuron classes and to more completely convert RID and CAN to an AIY-like fate. Such context-dependent restrictions of Lhx gene action have also been observed for the \textit{Drosophila Islet} gene and the \textit{C. elegans mec-3} gene. \textit{Islet} is necessary for expression of the dopaminergic and serotonergic phenotype of several ventral cord neurons, yet it is not sufficient to induce serotonergic phenotypes upon ectopic expression and is sufficient to induce dopaminergic phenotypes in only a small set of neurons (Thor and Thomas, 1997). Ectopic expression of the Lhx gene \textit{mec-3}, which is required to determine touch neuron fate, induces expression of a touch neuron marker in only one to 16 extra cells, all of which appear to contain a homeodomain protein, UNC-86, which acts together with MEC-3 in touch neurons (Duggan et al., 1998). The presence of UNC-86 may thus impose a requirement for ectopic MEC-3 activity that is comparable to the requirement of CEH-10 for ectopic TTX-3 activity.

\textit{ttx-3} does not appear to play a central role in the differentiation programs of other neurons besides AIY and possibly AIA. The expression of all tested differentiation markers of two types of sensory neurons that normally express \textit{ttx-3} is intact in \textit{ttx-3} mutants. Moreover, these sensory neurons appear structurally unaffected by loss of \textit{ttx-3} function. This is in striking contrast to AIY, in which the expression of all tested differentiation markers as well as axonal anatomy is severely affected in \textit{ttx-3} mutants. We thus consider it most likely that \textit{ttx-3} exerts a different regulatory impact in the cell fate specification of these sensory cell types. Owing to the absence of molecular markers as well as functional assays, the role of \textit{ttx-3} in AIA interneuron differentiation is unclear at this point. As the expression of the \textit{sra-11} and \textit{unc-17} fate markers is partly affected and as the AIA neurons appear to display anatomical defects in \textit{ttx-3} mutants, it is possible that \textit{ttx-3} is as crucial a differentiation regulator in AIA as it is in AIY.

\textbf{Function of \textit{ceh-23} in AIY differentiation} \textit{sra-11}, an orphan seven-transmembrane-receptor of unknown function, is most strongly expressed in the AIY and AVB neuron classes in L1 larvae, yet consistent and strong expression persists throughout adulthood in the AIY, but not the AVB neuron class. We have shown here that this AIY-specific maintenance mechanism requires \textit{ceh-23} gene activity. In contrast, the initiation of \textit{sra-11} expression requires \textit{ttx-3} but not \textit{ceh-23} activity. It is tempting to speculate that separable initiation and maintenance mechanisms introduce an additional level of regulation that could be subject to dynamic control. For example, CEH-23 protein activity could be post-transcriptionally regulated to allow for subtle and environmentally controlled changes in \textit{sra-11} expression during the life time of an animal.

The analysis of the role of \textit{ceh-23} and \textit{sra-11} in regulating aspects of AIY function will have to await a more complete understanding of the processes in which the AIY interneuron participates. While \textit{ceh-23}, and hence the maintenance of \textit{sra-11} expression, appear to have no impact on the function of AIY in processing thermosensory information, the complex patterns of synaptic connectivity of the AIY interneurons (White et al., 1986) suggest as yet unknown roles of AIY in processing and integration of various other types of sensory information. \textit{ceh-23} and \textit{sra-11} may have a role in determining these unknown aspects of AIY function.

\textbf{Gene regulatory interactions strongly depend on cellular context} The regulatory cascade of three homeobox genes and their target genes that we defined in the AIY interneurons shows intriguing aspects of both conservation and non-conservation in other neuron classes. Previously, Forrester et al., have shown that \textit{ceh-10} regulates \textit{ceh-23} in both AIY and CAN (Forrester et al., 1998). We show that in AIY this regulatory influence is exerted via \textit{ttx-3}; however, in CAN, the impact of \textit{ceh-10} on \textit{ceh-23} expression is independent of \textit{ttx-3} (inferred from both a lack of detectable \textit{ttx-3} expression in CAN and persistent \textit{ceh-23} expression in CAN in \textit{ttx-3} mutants). \textit{ceh-10} may induce the expression of a transcription factor in CAN that functions in \textit{ceh-23} regulation in an analogous fashion to \textit{ttx-3} in AIY. Adding to the complexity, the ADL sensory neuron class co-expresses \textit{ttx-3} and \textit{ceh-23}, yet \textit{ttx-3} has no
effect on ceh-23 expression in ADL (unlike in AY). Taken together, the promoter of the ceh-23 gene is likely to contain regulatory elements that are used differentially in distinct tissue types.

The largely non-overlapping patterns of expression of ceh-10, ttx-3 and ceh-23 and their complex patterns of regulatory relationship argue that none of these transcription factors alone is sufficient to impose specific differentiation aspects entirely on their own and thus point to a highly context-dependent co-factor requirement of each of these factors. Our findings illustrate the complexity of transcriptional regulation in the nervous system, and in particular, the complexity of interdependence of transcription factor action.

The context-dependent regulation of gene expression exemplified with the ceh-10, ttx-3 and ceh-23 cascade can also be illustrated at the level of the target genes of this transcription factor cascade, which is particularly intriguing as these are function genes that determine the individual properties of a neuron. For example, expression of the vesicular acetylcholine transporter UNC-17 marks the entire set of cholinergic neurons in C. elegans, which consists of at least 22 different neuron classes (Alfonso et al., 1993; Rand and Nonet, 1997). unc-17 gene expression is under control of TTX-3 exclusively in the AIY interneurons (and partly in AIA), and hence its expression must be controlled in a 'piece-meal' fashion by different transcription factors in different cholinergic cell types. Either the unc-17 promoter contains a transcription factor-binding site that is used by similar, yet differentially expressed members of a transcription factor class (e.g. a homeodomain-binding site that is used by homeodomain proteins that are present in distinct subsets of cholinergic neurons), or the unc-17 promoter displays a complex and highly modular structure that integrates different transcription factor inputs in distinct cholinergic cell types. Interestingly, cell-specific regulation of a neurotransmitter phenotype has also been described for the ttx-3 ortholog apertus in Drosophila. The Drosophila FMRF-NH2 neuropeptide gene is expressed in 44 neurons of the fly CNS, eight of which co-express apertus; in those eight cells FMRF-NH2 gene expression is under control of apertus, which in turn is expressed in about 100 CNS neurons (Benveniste et al., 1998). Thus, apertus is only required for FMRF-NH2 gene expression in a highly cell type-specific context.

C. elegans LIM homeobox genes: cell fate loss versus cell fate switch and transcription factor codes

The mutant phenotype of ttx-3 shows striking similarities and dissimilarities with the mutant phenotypes of other Lhx genes in C. elegans. In mec-3 mutant animals, touch sensory neurons have lost most if not all cell type-specific mechanosensory differentiation features, yet retain neuron-like features (Chalfie and Sulston, 1981; Duggan et al., 1998; Way and Chalfie, 1988), a phenotype that resembles the ttx-3 mutant phenotype described here. In contrast, lim-6 does not appear to be required for expression of most cell type-specific characteristics in a defined motoneuron and an interneuron (Hobert et al., 1999; O. Aurelio, and O. H., unpublished). The case appears more different with the C. elegans ortholog of the lhx6/8 subclass of Lhx genes, lim-4. In the absence of lim-4 function one class of cells that normally expresses lim-4, the AWB sensory neuron class, switches its fate in both structural and functional terms to that of another type of sensory neuron, the AWC class (Sagasti et al., 1999). Although it is possible that the AY neurons switch fate in ttx-3 mutants, we rather interpret the loss of cell fate marker gene expression, the abnormal appearance of AY and our failure to find ectopic marker gene expression as indication of a loss of cellular differentiation that is not necessarily accompanied by a cell fate switch.

These examples of differences of Lhx gene mutant phenotypes, i.e. complete loss of differentiation program (ttx-3), partial loss of differentiation program (lim-6) and a binary switch in differentiation program (lim-4) cannot, however, be taken as evidence for a specialization of function of members of specific Lhx subclasses. For example, while ttx-3 presumably affects the complete differentiation program of AY, we found no obvious impact of ttx-3 on sensory neuron differentiation. Additionally, while loss of lim-6 only partially affects the differentiation program of an interneuron and a motoneuron, it leads to a partial cell fate transformation in a specific sensory neuron class (Hobert et al., 1999). Taken together, individual C. elegans Lhx genes appear to have a highly context-dependent impact on neuronal differentiation.

We postulate that it is the information content of specific transcription factor codes that determines whether the loss of a Lhx gene in a given cellular context causes either a failure to differentiate or a switch to another neuronal cell fate. For example, in regard to lim-4 function in AWB it could be envisioned that the expression of the transcription factors a-e defines an 'abcde' code required for AWB development (with 'e' being lim-4), while the combination 'abcd' defines AWC fate. Loss of lim-4 hence leads to a switch in cell fate and the adoption of AWC fate. In the case of ttx-3, the transcription factor code 'fghjk' defines AIY fate ('k' being ttx-3); however, the combination 'fgh' has no specific coding potential and hence loss of ttx-3 simply leads to a failure to differentiate. Note that on first sight, our proposed classification of ttx-3 as a central regulator of AIY fate may argue that only a one letter code, 'k', may specify AIY fate; however, our demonstration of a strict context-dependency of ttx-3 function illustrates the need to postulate a multi-letter code. Thus, the outcome of a loss of a transcription factor in a given cell may be entirely predicted by the information content of the remainder of the transcription factor code.

A defined transcription factor code present in a postmitotic neuron at the stage of terminal differentiation may not however be the only determinant of the differentiation program of the cell; instead, in addition to the one-dimensional, spatial code of transcription factors present in a postmitotic neuron, there is also a second, temporal dimension to the differentiation program of a cell. That is, a neuron may have been exposed in its developmental history to certain determinants that have a perduring effect on the cell. Thus, an equivalent transcription factor code present in two postmitotic neurons that undergo terminal differentiation may be interpreted differentially in each cell, based on the individual history of the cell. Once the complete temporal and spatial profiles of transcription factor expression in any given neuron and its precursors is known, an achievement not too far away in a simple organism such as C. elegans, it may be possible to compute and model cell fates in a directed fashion.
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