A transcriptional regulatory cascade that controls left/right asymmetry in chemosensory neurons of C. elegans

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The molecular mechanisms of differential pattern formation along the left/right (L/R) axis in the nervous system are poorly understood. The nervous system of the nematode Caenorhabditis elegans displays several examples of L/R asymmetry, including the directional asymmetry displayed by the two ASE taste receptor neurons, ASE left (ASEL) and ASE right (ASER). Although bilaterally symmetric in regard to all known morphological criteria, these two neurons display distinct chemosensory capacities that correlate with the L/R asymmetric expression of three putative sensory receptor genes, gcy-5, expressed only in ASER, and gcy-6 and gcy-7, expressed only in ASEL. In order to understand the genetic basis of L/R asymmetry establishment, we screened for mutants in which patterns of asymmetric gcy gene expression are disrupted, and we identified a cascade of several symmetrically and asymmetrically expressed transcription factors that are sequentially required to restrict gcy gene expression to either the left or right ASE cell. These factors include the zinc finger transcription factor che-1; the homeobox genes cog-1, ceh-36, and lim-6; and the transcriptional cofactors unc-37/Groucho and lin-49. Specific features of this regulatory hierarchy are sequentially acting repressive interactions and the finely balanced activity of antagonizing positive and negative regulatory factors. A key trigger for asymmetry is the L/R differential expression of the Nkx6-type COG-1 homeodomain protein. Our studies have thus identified transcriptional mediators of a putative L/R-asymmetric signaling event and suggest that vertebrate homologs of these proteins may have similar functions in regulating vertebrate brain asymmetries.

Keywords: C. elegans; left/right asymmetry; transcription factor; homeobox

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The overall body plan of most adult animals is largely bilaterally symmetric [Ludwig 1932]. However, specific deviations from this bilateral symmetry are apparent at two different levels. First, individual organs such as the heart, stomach, and spleen are placed in a left/right (L/R) asymmetric manner in many organisms [Ludwig 1932]. Second, organs or morphological features that display an inherent pattern of bilateral symmetry can show defined deviations from symmetry. Such symmetry breakages are evident in the structure of seemingly bilaterally symmetric nervous systems of several species, from invertebrates to humans. In vertebrates, neuronal L/R asymmetry is evident in the different sizes of bilaterally positioned structures, such as the left and right temporal lobes of the human brain [Galaburda 1991] or the diencephalic habenular nuclei in several amphibian brains [Ludwig 1932]. More recently, L/R-specific gene expression patterns have been observed in the brains of several vertebrate species [Ramsdell and Yost 1998; Mercola and Levin 2001] and, in zebrafish, have been shown to correlate with the determination of L/R-specific features of diencephalic structures (Concha et al. 2000; Essner et al. 2000; Liang et al. 2000). Smaller sized invertebrate brains, in which individual cell types can be more easily identified and characterized, also reveal L/R asymmetries in otherwise largely bilaterally symmetric ganglia. These asymmetries include L/R-specific positioning of individual cells, L/R-specific cell death, and L/R-specific gene expression profiles [for review, see Hobert et al. 2002]. Of the two most widely used invertebrate model systems that are amenable to genetic studies, Drosophila melanogaster and Caenorhabditis elegans, neuronal L/R asymmetries have only been observed in C. elegans, thus making it the prime choice to genetically dissect the development of neuronal L/R asymmetry.

Evidence in vertebrates and the nematode C. elegans
suggests that bilateral neuronal structures may diversify with a L/R-specific bias as a means to increase the functional capacities of the nervous system. In humans, certain brain functions, such as language and attention, are highly lateralized [Davidson and Hugdahl 1994]. Nervous system functions in other vertebrate species, including rodents (Glick and Ross 1981) and fish [Miklosi et al. 1997], also display a lateral bias. In C. elegans, laterality has been observed in chemosensory capacities, specifically in the AWC and ASE neuron classes [Pierce-Shimomura et al. 2001, Wes and Bargmann 2001]. The AWC odorsensory neuron class consists of two neurons, AWC left and AWC right [AWCL and AWCR, respectively; Fig. 1], whose symmetries extend to many differentiated features of the neurons including cell position, axonal and dendritic morphology, outgrowth and placement, and synaptic connectivity (Fig. 1; White et al. 1986). In addition, both cells express similar sets of genes and are both required for chemotaxis to specific odorants [Bargmann et al. 1993]. However, a putative G-protein-coupled olfactory receptor, encoded by the str-2 gene, was found to be expressed asymmetrically in these neurons [Fig. 1; Troemel et al. 1999]. Induction of str-2 occurs stochastically through a calcium-signaling pathway in either the left or the right cell, but never in both [Troemel et al. 1999, Sagasti et al. 2001]. The functional significance of this stochastic distribution is demonstrated by the fact that mutants defective for str-2 asymmetry show odor-discrimination defects [Wes and Bargmann 2001]. The unbiased asymmetry exemplified through str-2 expression in either AWCL or AWCR is analogous to the unbiased asymmetry of many animal morphological features and hence can be termed “antisymmetry” [Palmer 1996]. The phenomenon of antisymmetry is contrasted with “directional asymmetry”, characterized by the sid-

Figure 1. Neural asymmetry in the AWC(L/R) and ASE(L/R) sensory neurons is genetically separable. The top panel is a schematic depiction of the anterior third of a worm, showing the anatomy and gene expression profiles of the AWC(L/R) odorsensory neurons [top left panels] and the ASE(L/R) gustatory neurons [top right panel]. The bottom panel shows a list of mutants that were tested for asymmetry defects in each neuron class. Results with mutants that affect asymmetric str-2 expression in AWC(L/R), monitored using the kyIs140 transgene, are taken from Troemel et al. (1999), with the exception of the cog-1, unc-37, and lin-49 results (our own results; the ASE defects are described in more detail in other figures). ASE(L/R) asymmetry was monitored using the lim-6 reporter transgene otsIs6 or otsIs114. A complete list of mutants tested for effect on asymmetric ASE(L/R) expression patterns is shown in Supplementary Table 1. References for the individual genes and alleles can be found in Troemel et al. (1999) and at http://www.wormbase.org. Notes: 1Ectopic lim-6::gfp expression is observed in a set of neurons other than ASE(L/R). 2The effect of the lin-49(ot74) null allele on str-2 expression (which in wild-type animals is 100% “one AWC on”, Troemel et al. 1999) is as follows: 38% “one AWC on,” 38% “no AWC on,” 14% “two AWC on,” 10% “more than two cells on” (n = 42). In contrast to these pleiotropic effects on AWC(L/R), the effect of lin-49 on lin-6 is qualitatively different; there is a stereotyped gain of ASER fate at the expense of the ASEL fate [see Fig. 5B]. Given this qualitative difference and also given the molecular identity of LIN-49 as a broadly expressed transcriptional cofactor with roles in multiple tissue types [see text], we do not consider the effect of lin-49 on AWC and ASE as specific evidence that the AWC and ASE asymmetries are mechanistically related.
edness of morphological features not being randomly distributed within a population, but strongly biased to one side of each individual [Palmer 1996]. A clear case of directional asymmetry is displayed by the main class of C. elegans gustatory neurons called ASE. Like the AWC odorsensory class, the ASE neuron class consists of two bilaterally symmetric cells, called ASEL (ASE left) and ASER (ASE right), that are symmetric with regard to cell position, axonal and dendritic morphology, and patterns of synaptic connectivity [Fig. 1; White et al. 1986]. However, three putative sensory receptors of the guanylyl cyclase receptor family are asymmetrically expressed in ASEL [gcy-6 and gcy-7] or ASER [gcy-5, Fig. 1; Yu et al. 1997]. Laser ablation of ASEL or ASER revealed that these asymmetric gene expression profiles correlate with functional asymmetry of the two neurons, each neuron is responsible for detecting a distinct class of water-soluble chemicals [Pierce-Shimomura et al. 2001]. The functional significance of this L/R separation of chemosensory capacities was revealed through the analysis of lim-6 mutant animals in which the ASEL neuron, which normally senses sodium, but not chloride, now adopts the chloride-sensing feature of the ASER neuron [Hobert et al. 1999; Pierce-Shimomura et al. 2001]. Hence, in lim-6 mutant animals, ASEL has the capacity to sense both sodium and chloride. These animals fail to effectively discriminate between the two chemicals, that is, they fail to sense one ion in the presence of the other [Pierce-Shimomura et al. 2001]. The L/R separation of the chemosensory capacities of the ASE neurons in wild-type animals therefore increases the chemosensory capacities of the animals.

What are the cellular and molecular mechanisms that lead to functional diversification of ASEL and ASER? We have previously shown that the lim-6 LIM homeobox gene is required to repress expression of the ASER-specific guanylyl-cyclase gene gcy-5 in ASEL. However, lim-6 is only one of presumably many factors that establishes asymmetry because first, lim-6 itself is already asymmetrically expressed in ASEL but not in ASER [Hobert et al. 1999] and second, ASER-specific expression of the guanylyl cyclase genes gcy-6 and gcy-7 is unaffected in lim-6 null mutants [Hobert et al. 1999]. In order to elucidate the molecular mechanisms required to restrict expression of lim-6 and the guanylyl cyclase genes in an asymmetric manner to just one of the two ASE neurons, we have undertaken a genetic screen to uncover mutants that show symmetrization of normally asymmetric ASE[L/R] features and report here the molecular identity of a subset of these mutants. Our study thus provides novel insights into the as yet poorly understood mechanisms of L/R diversification in the nervous system.

Results

L/R asymmetry in odorsensory and gustatory neurons is genetically separable

We first tested whether the determination of directional asymmetry in the ASE[L/R] gustatory neurons shares mechanistic similarities with the antisymmetric gene expression pattern of str-2 in the AWC[L/R] odorsensory neurons. Mutants that were previously shown to affect antisymmetry of str-2 gene expression in AWC[L/R] fall into three broad categories.

First, mutations that disrupt axon guidance cause str-2 asymmetry defects [Troemel et al. 1999]. This defect has been attributed to a failure of the establishment of a direct cell–cell contact between the axons of AWCL and AWCR, which normally meet at the dorsal midline, run in close proximity to one another, and make reciprocal synaptic contacts with one another [White et al. 1986]. Although making no reciprocal synaptic contact [White et al. 1986], the axons of ASEL and ASER also run in close proximity to one another after meeting at the dorsal midline [D. Hall and O. Hobert, unpubl.] Disruption of these contacts through the use of the same set of axon guidance mutants that disrupt asymmetric str-2 expression in AWCL/R has, however, no effect on asymmetric reporter gene expression in ASE[L/R] (Fig. 1; Supplementary Table 1).

Second, mutations that disrupt calcium signaling and mitogen-activated protein kinase (MAPK) signaling within AWC[L/R] cause str-2 asymmetry defects [Troemel et al. 1999; Sagasti et al. 2001]. The same set of signaling mutants has no effect on asymmetric reporter gene expression in ASE[L/R] (Fig. 1; Supplementary Table 1).

Third, antisymmetric str-2 expression in AWC[L/R] was found to be affected in mutants that disrupt olfactory sensory processing [Troemel et al. 1999]. We tested whether more than a dozen mutants that disrupt taste perception affect ASE[L/R] asymmetry. With the exception of che-1 (described following), we found this not to be the case [Fig. 1; Supplementary Table 1].

Last, as we will describe following, we have identified mutant alleles in which ASE[L/R] asymmetry is affected, but AWC[L/R] is not [Fig. 1]. Consistent with their distinct appearance [antisymmetry vs. directional asymmetry], we conclude that the mechanisms of establishment of AWC[L/R] and ASE[L/R] asymmetry are genetically separable.

Identification of genes that affect ASE[L/R] asymmetry

Besides the mutant backgrounds mentioned earlier, we tested a variety of candidate genes for an effect on asymmetric expression of ASEL markers, including lin-12/Notch, receptor tyrosine kinase (RTK), transforming growth factor (TGFβ)-, and Wnt-signaling mutants and various transcription factors and other known patterning mutants and did not observe any defects [Supplementary Table 1]. Given the previously reported impact of TGFβ-like signaling on the determination of L/R asymmetry in vertebrate organ and brain development [Ramsdell and Yost 1998; Mercola and Levin 2001], the absence of a defect in null mutants of the dal-4 gene, which codes for the sole type II TGFβ-receptor protein in the C. elegans
genome [Estvez et al. 1993; Ruvkun and Hobert 1998],
is of interest because it points to a different mechanism
of regulation of L/R asymmetry.
Using transgenic reporter strains that express green
fluorescent protein (gfp) exclusively in ASEL [lim-6::gfp and
gcy-7::gfp], we then conducted unbiased genetic
screens for mutants that display defects in asymmetric
ASE marker gene expression [see Materials and Meth-
ods]. Mutants derived from these screens were examined
for defects in asymmetric expression of three ASE asym-
metry markers, lim-6::gfp and gcy-7::gfp for ASEL and
gcy-5::gfp for ASER. Because two ASEL markers, lim-
6::gfp and gcy-7::gfp, show identical behaviors in all
mutant backgrounds tested, we have not included a third
ASEL marker, gcy-6::gfp, in our analysis but assume that
it behaves similarly to lim-6::gfp and gcy-7::gfp. Cons-
istent with this notion, lim-6, gcy-7 and gcy-6 contain
significant patches of sequence similarity in their cis-
regulatory regions [data not shown].
We retrieved three classes of mutants from our screen.
In class I and class II mutants, the overall identity of the
ASE neurons, as assessed by cell position, axon morphol-
yogy, and bilaterally symmetric gene expression profiles
is unaffected. In class I mutants, however, the ASEL
markers lim-6::gfp and gcy-7::gfp are expressed in both
ASEL and ASER, and gcy-5::gfp expression is concomi-
tantly lost in ASER [“two ASEL”-phenotype, see Fig. 2
for examples]. In class II mutants, lim-6::gfp and gcy-
7::gfp fail to be expressed in ASEL, and there is concomi-
tant ectopic expression of gcy-5::gfp in ASER [“two
ASER”-phenotype, see Fig. 5, below, for examples]. Last,
in class III mutants, the ASE neurons are generated but
lack the expression of several identity-determining
markers, including all three ASEL- and ASER-specific
asymmetry markers as well as bilaterally symmetric
markers [see Fig. 8, below, for examples; Fig. 9A, below,
shows a schematic summary of all mutant phenotypes in
single and double mutants]. We termed mutants from
the first two classes “lsy” mutants [pronounced “lousy”],
for lim-6 symmetry mutant. We also noted that
lsy mutants do not disrupt the L/R asymmetric place-
ment of a specific unilateral neuron [RIS] or the L/R
asymmetric migration of the Q neuroblasts [data not
shown].
We mapped most lsy alleles to specific chromosomal
intervals. A combination of complementation testing and
comparison of map position allowed us to conclude that
we retrieved a total of 11 complementation groups,
five displaying the class II phenotype (“two ASEL”), an-
other five displaying the class I phenotype (“two ASER”),
and one displaying the class III phenotype (“ASEL/R
off”). One more class I mutant has not yet been ordered
into a complementation group. Several of the comple-
mentation groups are represented only by single alleles,
demonstrating that our screening efforts have not yet
reached saturation. In this paper, we will describe the
molecular characterization of two complementation
groups that display the class I lsy phenotype (cog-1 and
unc-37), two complementation groups that display the
class II lsy phenotype [che-1 and lin-49], and the single
complementation group that displays the class III pheno-
type [che-1].

Class I mutants (‘two ASEL’) affect the
ASE(L/R)-expressed COG-1 and UNC-37
transcription factors
The four class I lsy mutants ot28, ot38, ot62, and ot59
define two complementation groups [Fig. 2]. Through
single-nucleotide polymorphism (SNP) mapping,
complementation testing, transformation rescue, and al-

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**Figure 2.** Mutations resulting in a symmetric, “two ASEL”
phenotype [class I phenotype]. (A) Expression patterns of gcy-
7::gfp, lim-6::gfp, and gcy-5::gfp in wild-type, cog-1(ot28), and
unc-37(e262) mutant backgrounds. Adult animals are shown.
Both gcy-7::gfp and lim-6::gfp are derepressed in ASER in unc-
37 and cog-1 backgrounds, whereas gcy-5::gfp expression is
lost in ASER. Arrows denote the excretory gland cell that expres-
ses lim-6::gfp. (B) Quantification of the effects of cog-1 and unc-37
on ASE asymmetry. Animals were scored as adults [with the
exception of ot59, which, because of their lethality, were scored
as L1s]. Note that derepression of lim-6 in ASER is always fol-
lowed by a concomitant loss of gcy-5 expression.
described shown to code for a homeobox gene orthologous to the ortholog of Groucho, corepressor Groucho (Muhr et al. 2001). The engrailed homolog (eh1) domain with the transcriptional orthologs of COG-1 interact through the conserved lsy phenotype [Fig. 2B].

In vitro binding assays revealed that the vertebrate orthologs of COG-1 interact through the conserved engrailed homolog [eh1] domain with the transcriptional corepressor Groucho [Muhr et al. 2001]. The *C. elegans* ortholog of Groucho, unc-37, maps to a chromosomal region on linkage group I [Pflugrad et al. 1997], to which we mapped another asymmetry mutant, *ot59*, with a *lsy* phenotype similar to our *unc-37* alleles [Fig. 2]. *ot59* animals also displayed a characteristic *unc-37*-like locomotory defect. We sequenced the *unc-37* gene in *ot59* mutant animals and found an early splice site mutation [Fig. 3A]. The canonical allele of *unc-37*, *e262*, also shows a class I *lsy* phenotype, which can be rescued through the introduction of the wild-type *unc-37* locus [Fig. 4A].

cog-1 and unc-37 interact genetically

UNC-37 has recently been shown to interact with the engrailed homology [eh1] domain of the UNC-4 homoeodomain protein [Winnier et al. 1999]. The eh1 domain is also conserved in COG-1 [Fig. 2A]. Moreover, the vertebrate orthologs of cog-1 and unc-37, Nkx6.1 and Grg4/Groucho, directly interact in vitro through the eh1 domain [Fig. 2A; Muhr et al. 2001], suggesting that COG-1 and UNC-37 may also directly interact to affect ASE asymmetry. To corroborate this notion, we examined a potential genetic interaction between cog-1 and unc-37. Lowering the dose of either cog-1 or unc-37 through placing a wild-type copy of the respective gene over a hypomorph allele has no effect on asymmetry [0 out of 115 cog-1(*ot28*)/+ animals show ectopic gcy-7: Gal4::GFP expression in ASER, 0/84 unc-37(*e262*)/+ animals show defects]. If, however, the dosage of both genes is simultaneously reduced in a transheterozygous state, a significant asymmetry defect becomes obvious [26/95 unc-37(*e262*)/+ cog-1(*ot28*) animals show ectopic gcy-7: Gal4::GFP expression in ASER]. Later we report that cog-1 and unc-37 act in a similar cell to affect asymmetry, thus leading us to conclude that, like their vertebrate orthologs, COG-1 and UNC-37 are likely to physically associate to regulate asymmetric patterns of gene expression.

unc-37 and cog-1 expression in the ASE neurons

Consistent with the ubiquitous expression of its vertebrate and fly orthologs, *unc-37*/Groucho was previously reported to be broadly expressed [Pflugrad et al. 1997], however, its expression was not specifically examined in individual head neurons. We therefore examined the expression of *unc-37* in ASE[L/R] through the use of an UNC-37::GFP translational reporter [Kelly et al. 1997], which we found to rescue the asymmetry defects of *unc-37* mutants [Figs. 3B, 4A]. A red fluorescent protein *rfp*-expressing transgene, *otIs131*, which is exclusively expressed in larval ASE[L/R] neurons, shows co-fluorescence with the unc-37::gfp reporter signal [Fig. 3B], thus demonstrating unc-37 expression in ASEL and ASER.

A cog-1::gfp reporter gene fusion was previously reported to be expressed in three classes of head sensory neurons, including ASEL and ASER [Palmer et al. 2002]. Using previously described reporter lines as well as newly constructed reporter lines, we made the intriguing observation that all transgenic lines tested show significantly higher levels of cog-1 expression in ASER versus ASEL [Fig. 3B,C]. Higher levels of expression of cog-1 can be observed with a rescuing cog-1::gfp reporter gene fusion, which contains the complete coding region of cog-1, as well as with reporter gene fusions in which only the promoter of cog-1 is fused to *gfp* [Fig. 3C]. This observation indicates that differential expression of cog-1 in ASER versus ASEL is mediated via the transcriptional level.

cog-1 and unc-37 act in ASER and through lim-6 to affect gcy-5 expression

We first focused on the effects of cog-1 and unc-37 mutations in ASER, which loses gcy-5 expression [ASER marker] and gains lim-6 expression [ASEL marker] in the respective mutants. First, we tested whether cog-1 and unc-37 function is cell autonomous. We found that cDNAs of unc-37 and cog-1 driven by a postmitotic, ASER-specific transcriptional regulatory element derived from the gcy-5 locus rescue the respective mutant phenotypes [Fig. 4A], indicating that these two genes may act postmitotically in ASER to repress ASER-specific features.

Second, we examined whether the effect of cog-1 and unc-37 on asymmetric gcy-5 expression is mediated through the regulation of the homeobox gene *lim-6*, *lim-6*, which is normally expressed exclusively in ASEL, is required to repress gcy-5 expression in ASER [Hobert et al. 1999]; in cog-1 and unc-37 mutants, *lim-6* is ectopically expressed in ASER, and gcy-5 expression is concomitantly lost in ASER. To ask whether this loss of gcy-5 expression is due to ectopic *lim-6* expression, we eliminated *lim-6* in an unc-37 mutant and in a cog-1 mutant background. We find that in both cog-1; *lim-6* and unc-37; *lim-6* double mutants, gcy-5 expression in ASER reappears [Fig. 4B]. Hence, in wild-type animals, cog-1 and unc-37 allow gcy-5 to be expressed through inhibiting the expression of the gcy-5 repressor *lim-6*. The lack of a perfect correlation between gain of *lim-6* expression [e.g., 53% of unc-37 mutant animals gain *lim-6* in ASER, but only 32% concomitantly lose gcy-5 expression, Fig. 2B] may be a reflection of a need for a threshold level of ectopic *lim-6* expression to achieve gcy-5 repression; this level may not be reached in all animals.
Figure 3. Mutations in cog-1 and unc-37, two transcription factors expressed in ASE(L/R), cause ASE asymmetry defects. (A) Schematic depiction of the UNC-37 and COG-1 protein structure (drawn to scale) with mutant alleles noted. The blowup of the COG-1 sequence depicts a motif similar to the eh1 domain [alignment extended from Muhr et al. (2001)]. Colored residues indicate conservation to the Engrailed protein [red, identity; blue, conservative substitution], underlining indicates identity (in >50% of aligned sequences) among the NK-type protein that cannot be observed in the Engrailed protein. In contrast to the previously characterized cog-1 alleles sy607 and sy275, which still produce progeny, the ot38 and ot62 alleles, the latter of which only affects the cog-1 splicing form [A], are completely sterile. ot28 animals are fertile. The lsy phenotype of ot62, but not any other cog-1 allele, is dominant (23% of ot62+/+ animals are lsy, n = 22). cog-1(ot62) animals may produce a truncated COG-1A protein that contains only its transcriptional repressor domain, the eh1 domain, but not its DNA-binding domain. In heterozygous animals, this truncated protein may interfere in a dominant-negative manner with the activity of the wild-type copy of the COG-1A protein. (B) Expression of a translational UNC-37::GFP fusion protein (left panel; Kelly et al. 1997) and a translational COG-1::GFP fusion protein (right panel; transgenic line, sy1663; Palmer et al. 2002) in the ASE neurons in midlarval stage animals [white arrowheads]. ASEL and ASER are marked with the ASE(L/R)-expressed reporter rfp transgene otIs131 [Ex[gcy-7::rfp]] or otEx445 [Ex[gcy-7::rfp]]. Asterisks denote gut autofluorescence. (C) Quantification of cog-1 reporter gene expression in different transgenic lines in ASEL and ASER. The top five lines carry transcriptional reporter fusions that contain the promoter of the cog-1 gene. syIs73 is a chromosomally integrated array [Palmer et al. 2002]. The bottom five lines carry translational reporter fusions that contain the promoter as well as all coding sequences of cog-1. otEx1006 rescues the cog-1 mutant phenotype [Fig. 4A; other lines were not tested for rescue]. ASER > ASE indicates stronger gfp fluorescence in ASE compared with ASE. Numbers in parentheses indicate complete absence of fluorescence in ASE. In theory, the occasional, less consistent expression of cog-1 in ASEL could be a reporter gene artifact, explained through the titration of an ASEL-specific negative regulator of cog-1 expression. However, genetic evidence described in the text indicates that, in specific mutant backgrounds [ceh-36, lin-49], a function for cog-1 in ASE is revealed, demonstrating that low levels of cog-1 activity are indeed present in ASE. All animals contained gcy-7::rfp reporters in the background [otIs131 or otEx445], were photographed at midlarval stages in which gcy-7::rfp reporter is expressed in ASEL and ASER, and were scored for the gfp phenotype as gravid adults.

After having shown that lim-6 is required to repress gcy-5 expression, we next tested whether lim-6 alone is sufficient to repress gcy-5 expression. To this end, we expressed lim-6 in both ASEL and ASER (and in all other neurons of the nervous system, using the unc-119 promoter; see Materials and Methods) in a lim-6 null mutant background. We found that, in these transgenic animals, the loss of gcy-5 repression in ASEL is rescued, confirming that this promoter produces sufficient levels of a functional LIM-6 protein [Fig. 4C]. However, in none of the animals in which the repression of gcy-5 in ASE is reestablished can we observe a concomitant repression of gcy-5 expression in ASER. The requirement but lack of sufficiency for lim-6 function suggests that the ectopic lim-6 expression observed in cog-1 and unc-37 mutants is necessary, but not sufficient, to repress gcy-5 expression. Other factors must similarly be derepressed in ASER in cog-1 and unc-37 mutants to allow lim-6 to repress gcy-5 expression.

Overexpression of cog-1 can convert ASEL to ASER

We next asked whether raising the levels of cog-1 in ASEL may be sufficient to repress ASE features [lim-6] and allow ASER features [gcy-5] to appear. We used two approaches: First, we generated multicopy arrays of the cog-1 locus and second, we expressed a cog-1 cDNA under control of the gcy-7 promoter, which is active in ASEL and ASER embryonically and becomes restricted to ASEL postembryonically. We found that transgenic wild-type animals expressing either of these two constructs show repression of lim-6 expression in ASEL and a concomitant gain of the normally ASER-specific marker gcy-5 expression in ASEL [Fig. 4D].
Neuronal left/right asymmetry

Figure 4.  cog-1 and unc-37 act autonomously to repress ASEL fate via repression of lim-6.  (A) cog-1 and unc-37 act cell autonomously. Transformation rescue data of the unc-37 and cog-1 mutant defects are shown. Numbers below bars indicate independent transgenic strains. “% defective” refers to the absence of gcy-5::gfp expression in ASER, assessed with the nts1 [Is[gcy-5::gfp]] integrated transgene. “cog-1::gfp” [otEx1006, otsEx1007; see Materials and Methods] and “unc-37::gfp” [Kelly et al. 1997] are translational gfp fusions in which the respective genomic locus is fused to gfp. cog-1::gfp was injected at 10 ng/µL and gcy-5::cog-1 at 2 ng/µL [lines 1 and 2], 5 ng/µL [lines 3 and 4], and 50 ng/µL [lines 5 and 6]. gcy-5::unc-37 was injected at 50 ng/µL. rol-6 was the injection marker. Control lines have the cog-1 or unc-37 coding region replaced with gfp and were generated at 50 ng/µL injected DNA. We explain the ability to rescue gcy-5 expression through supplying cog-1 and unc-37 under control of the gcy-5 promoter by the gcy-5 promoter not being entirely shut off in the respective mutants. (B) cog-1 and unc-37 act through lim-6. Loss of gcy-5::gfp expression [monitored with nts1] in ASER in cog-1 and unc-37 is suppressed by removing lim-6 activity. (C) lim-6 is not sufficient to repress gcy-5 expression. gcy-5::gfp [nts1] is in the background of all strains. “lim-6r” is a rescuing lim-6 genomic fragment previously described to rescue other lim-6 mutant defects (Hobert et al. 1999) and was injected at 20 ng/µL unc-37; that is, they display a “two ASER” phenotype [Fig. 5A,B]. Bilaterally symmetric features of ASEL/L/R, such as the expression of the flip-6 gene, are unaffected in class II mutants [data not shown], suggesting that these mutants specifically disrupt the asymmetric gene expression programs. We will first describe two of these class II genes and in the next section we will describe their genetic interactions with cog-1 and unc-37.

Lin-49: Through SNP mapping, complementation testing, allele sequencing, and transformation rescue, we found that the three recessive ots69, ots74, and ots78 alleles are mutations in the lin-49 gene, which codes for a predicted nuclear protein with two plant homeodomain (PHD)-finger domains and a bromodomain (Chamberlin and Thomas 2000). The PHD-finger domains, found in many chromatin-associated proteins [Aasland et al. 1995], and the bromodomain, an acetyllysine-binding domain [Dyson et al. 2001], indicate that the LIN-49 protein may be a general transcriptional cofactor involved in chromatin remodeling. Consistent with this notion, lin-49 was reported to be broadly expressed and shown to affect the development of several tissue types including the gut, the egg-laying system, and male mating structures [Chamberlin and Thomas 2000]. We corroborated the broad and possibly ubiquitous expression of lin-49...
using a gfp fusion construct that rescues the mutant phenotype [Fig. 6C].

ceh-36: Through SNP mapping, allele sequencing, and transformation rescue, we found that ot79 is an allele of the previously uncharacterized ceh-36 gene, one of three orthodenticle-type homeobox genes predicted in the C. elegans genome [Fig. 6A,B, Ruvkun and Hobert 1998]. The ot79 allele is completely recessive but unlikely to be a null allele because the truncation introduced by the premature stop codon still leaves the homeodomain intact [Fig. 6A]. A ceh-36::rfp fusion construct that is capable of rescuing the mutant phenotype [Fig. 6C], as well as a fusion of the ceh-36 upstream regulatory region to gfp, showed exclusive expression in two pairs of head sensory neurons in postembryonic animals, one being ASE[L/R] [Fig. 6D; A. Lanjuin and P. Sengupta, pers. comm.].

Because both lin-49 and ceh-36 mutants have similar effects on lim-6 and gcy gene expression, we considered whether they may do so in a sequential manner through activating each other’s expression. Since we consider it highly unlikely that a broadly expressed transcriptional cofactor is under control of the 2-neuron-specific transcription factor ceh-36, we only tested the possibility that ceh-36 expression may be regulated by lin-49. We found that a ceh-36::gfp reporter construct is normally expressed in lin-49 null mutants; it is also unaffected in cog-1 and unc-37 mutants (data not shown). Following we present genetic epistasis data that indicate that ceh-36 and lin-49 show very similar patterns of interaction with other transcription factors, suggesting that CEH-36 and LIN-49 may act together, possibly in a complex analogous to COG-1 and UNC-37.

The cog-1/unc-37 repressor complex antagonizes the ceh-36/lin-49-mediated activation of lim-6 expression

We have shown that lin-49 and ceh-36 are required for lim-6 and gcy-7 expression in ASE L. Yet ceh-36 [and the
ubiquitously expressed lin-49 gene) is expressed in both ASE and ASER. What prevents ceh-36 and lin-49 from activating lim-6 expression in ASER? One possibility is that their activity is antagonized in ASER by the transcriptional repressors cog-1 and unc-37. We tested this hypothesis by asking whether lowering the activity of cog-1 and unc-37 now allows lin-49 and ceh-36 to activate lim-6 expression in ASER. To this end, we constructed a variety of double mutant animals. We find that ectopic expression of lim-6 in ASER in cog-1 and unc-37 mutants requires the activity of both lin-49 and ceh-36 because, in either double mutant combination, ectopic lim-6 expression in ASER is diminished (Fig. 7A).

The model of an antagonism between cog-1/unc-37 and ceh-36/lin-49 is further corroborated by two experiments. First, as described earlier, raising the levels of cog-1 in ASER (which normally expresses lower levels of cog-1 than does ASER) counteracts the normal activation of lim-6 by ceh-36 and lin-49 in ASER and hence leads to a loss of lim-6 expression (Fig. 4D; see also model in Fig. 7C). Second, we find that reduction of ceh-36 or lin-49 activity does not merely lead to a loss of transcriptional activation of lim-6 in ASER but, to the contrary, leads to a cog-1-dependent repression of lim-6. This is because, if we lower cog-1 activity in a ceh-36 or lin-49 hypomorphic background, lim-6 expression in ASER is at least partially, if not completely, restored (Fig. 7B,C); a concomitant repression of gcy-5 in ASER that correlates with restored lim-6 expression can also be observed; data not shown). This experiment corroborates our observation of low levels of cog-1 expression in ASER, whose repressive activity in wild-type animals seems to be antagonized by ceh-36 and lin-49 activity; lowering of ceh-36 and lin-49 activity hence allows cog-1 to repress lim-6.

Also consistent with the notion of cog-1/unc-37 and ceh-36/lin-49 acting in parallel rather than sequentially, we find that L/R asymmetric expression of a cog-1 reporter gene construct is unaffected in ceh-36 and lin-49 mutants and that ceh-36 reporter gene expression in a cog-1 and unc-37 mutant background is unaffected (data not shown).

One way to fit this genetic data into a molecular model is to suppose that a COG-1/UNC-37 repressor...
We found that all class III mutants fall into a single complementation group that is allelic to the che-1 locus. We have described earlier a set of genes that act as positive regulators of gcy-7 and lim-6 in ASEL, with the latter factor being a repressor of the ASER marker gcy-5. But what factor is required for the activation of gcy-5 expression in ASER? In cog-1 and unc-37/groucho mutants, gcy-5 expression in ASER is lost. Groucho has previously been shown to be able to convert transcriptional activators, such as Runt or Dorsal, into repressors [Fisher and Caudy 1998]. It could have thus been envisioned that cog-1 is an unc-37/groucho-independent activator of gcy-5 expression and an unc-37/groucho-dependent repressor of lim-6. However, our genetic interaction data show that this is not the case, because we can restore gcy-5 expression in cog-1 mutants if we remove the lim-6 gene. cog-1 thus “activates” gcy-5 expression through repressing the gcy-5 repressor lim-6.

Activation of gene expression through counteracting repression automatically invokes the existence of a basal transcriptional activation mechanism. We considered it possible that class III mutants that we have retrieved from our screen may shed light on the issue of transcriptional activation mechanism. We hypothesized that both proteins act in a common transcriptional activation complex because both genes behave indistinguishably in genetic interaction tests. Loss of either causes the same effects on the expression of downstream genes, they are both required for ASEL-marker expression in ASER in the absence of cog-1 or unc-37, and their reduction of activity in ASER is compensated by removal of cog-1. The domain structures of the two proteins are also supportive of a role for these proteins in a single transcriptional activation complex in which CEH-36 may provide the DNA binding specificity and LIN-49 the connection to the chromatin remodeling activities required for gene activation.
marker is still expressed in ASE(L/R) and because the anatomy of ASE is only partially affected in che-1 mutants [Lewis and Hodgkin 1977; Uchida et al. 2003]. We propose that che-1 is a permissive transcriptional regulator, located at the top and at the lower levels of a transcriptional regulatory cascade required to initiate asymmetric profiles of gene expression in ASEL and ASER.

Discussion

Cellular diversification along the L/R axis is a common feature in nervous systems as diverse as nematodes and humans, yet its molecular basis is still poorly understood [Hobert et al. 2002]. The genetic amenability of C. elegans enabled us to identify a set of proteins involved in determining neuronal L/R patterning, most of which are highly conserved across phylogeny. In C. elegans, these factors serve to eventually segregate the expression of putative sensory receptors encoded by the gcy genes into otherwise largely bilaterally symmetric types of cells. The separation of distinct sensory capacities into two separate, yet bilaterally symmetric cells, each of which has a similar set of downstream synaptic partners [White et al. 1986], is a prerequisite for the capacity of worms to appropriately discriminate between distinct sensory inputs and hence to navigate in a complex sensorial environment [Pierce-Shimomura et al. 2001]. The increase in the discriminatory sensory potential through asymmetric chemoreceptor gene expression is not only a feature of the gustatory ASE neurons and ASER neurons, but also of the olfactory AWCL and AWCR neurons [Wes and Bargmann 2001]. Despite their similar purpose, our genetic analysis points to the distinctiveness of the molecular mechanisms of establishment of AWCL and ASE asymmetries. Although in the former case, cell–cell contact, calcium, and MAPK signaling are required to establish antisymmetric gene expression pattern [Troemel et al. 1999; Sagasti et al. 2001], none of these events is required to establish directional ASE asymmetry.

A molecular model that sheds some light onto the L/R asymmetric segregation of chemosensory receptor expression has emerged from our genetic studies and can be summarized as follows [Fig. 9]. The Zn finger transcription factor CHE-1 acts at the top of the regulatory hierarchy to determine subtype-specific and bilateral-symmetric features of ASE, including the expression of terminal differentiation markers such as the neuropeptide gene \( \text{gfp} \), cyclic nucleotide ion channels, and orphan serpentine receptors [Uchida et al. 2003; this paper]. CHE-1 also triggers either directly or indirectly the expression of factors that are required to determine the L/R asymmetric state of ASE in ASE, including \( \text{cog-1} \), \( \text{ceh-36} \), and \( \text{lim-6} \). These factors serve to modulate what we consider an “ASE default state,” which is defined by the expression of \( \text{gcy-5} \). This default state is also revealed through laser ablation studies, demonstrating that the removal of putative signaling cells causes ASE to convert to the ASE state [R.J. Johnson Jr. and O. Hobert, unpubl.] features of this default state, such as \( \text{gcy-5} \) ex-

Figure 8. Mutations in the che-1 locus eliminate adoption of the ASE(L/R) fate [class I phenotype]. (A) ASE(L/R) expression of \( \text{gcy-7:gf} \) (otIs13), \( \text{lim-6:gf} \) (otIs114), \( \text{gcy-5:gf} \) (otIs1), and \( \text{flp-6:gf} \) (otIs125) in adult wild-type and adult che-1 (ot27) mutant animals. ASE-specific expression of all reporters is lost in che-1 mutants. Arrows in the “\( \text{lim-6:gf} \)” panel denote the excretory gland cell that expresses \( \text{lim-6:gf} \) and the ADF or AFD neuron class in the \( \text{flp-6:gf} \) panel, note that \( \text{gf} \) expression in neither of these cell types is affected in che-1 mutants. Asterisks denote gut autofluorescence. (B) Quantification of che-1 effects on ASE gene expression patterns. (C) Schematic CHE-1 protein structure (drawn to scale), denoting the position of mutant alleles retrieved from our screen. The deletion breakpoints in \( \text{ot66} \) have not been sequenced but are inferred by PCR.
expression, may be directly induced by CHE-1. After CHE-1 has induced CEH-36 and COG-1 expression, a putative CEH-36/LIN-49 complex activates expression of at least two factors, a gcy gene (gcy-7) that defines ASE properties and a transcription factor (lim-6) that prevents the ASER-specific gcy-5 gene from being expressed (Fig. 9). Although the bilaterally symmetrically expressed CEH-36/LIN-49 proteins (which may in analogy to COG-1 and UNC-37 be acting in a complex) are capable of inducing lim-6 and gcy-7 expression in both ASE and ASER, they are prevented from doing so in ASER through the activity of the COG-1/UNC-37 repressor complex, which thus helps preserve the ASER default state [Fig. 9]. The activity of COG-1/UNC-37 antagonizes CEH-36/LIN-49 activity only in ASER because of higher levels of COG-1 protein in ASER. Consequently, raising the levels of COG-1 in ASE can overcome the CEH-36/LIN-49-mediated induction of ASE cell fate, causing the adoption of ASER fate.

Our studies have revealed two basic transcriptional regulatory principles in the novel context of L/R asymmetry determination. First, we have shown that the expression of ASER fate (gcy-5 expression) is mediated through a series of repressive transcriptional interactions.
Neuronal left/right asymmetry

Materials and methods

Transgenic reporter lines

All strains were grown at 20°C and scored at room temperature as gravid adults if not otherwise indicated.

otIs6 and otIs114: Is[lim-6prom::gfp; rol-6(d)]. Both integrants derive from a previously described extrachromosomal line (Hobert et al. 1999). In contrast to previous nomenclature [Hobert et al. 1999], we will for the sake of simplicity from here on refer to “lim-6prom::gfp” as “lim-6::gfp.”

otIs3: Is[gcy-7::gfp; lin-15(+)]. In mid-embryogenesis, otIs3 is initially expressed in both ASE and ASER; expression becomes restricted to ASER in late embryogenesis. This integrant derives from an extrachromosomal line described in Yu et al. (1997).

otIs125: Is[dpy-6::gfp]. The extrachromosomal line from which this integrant was derived was a gift from C. Li.

otIs131: Is[gcy-7::rfp; rol-6(d)], derived from otExd45 (see following).

ntIs1: Is[gcy-5::gfp; lin-15(+)] [a gift from S. Lockery; derived from an extrachromosomal line described in Yu et al. (1997)]. Expression of gfp is turned on exclusively in ASER in embryogenesis after the generation of ASER.

ktys140: Is[atr-2::gfp; lin-15(+)] [Troemel et al. 1999], a 5′ fusion (i.e., containing only regulatory sequences upstream of the start codon).

otEx445: Ex[gcy-7::rfp; rol-6(d)], a 5′ fusion. Because of the delayed maturation of the RFP protein (compared with the GFP protein expressed from the otIs3 transgene mentioned earlier), GCY-7::RFP expression from this array can be observed in both ASE and ASER until midlarval stages and becomes restricted to ASE thereafter.

otEx862: Ex[cel-36prom::gfp; rol-6(d)], a 5′ fusion.

otEx1030, otEx1031: Ex[cel-36::rfp; rol-6(d)], a translational fusion (i.e., containing upstream regulatory sequences as well as all exons and introns of the locus).

otEx863: Ex[lim-49prom::gfp; rol-6(d)], a 5′ fusion.

otEx1025–1029: Ex[lim-49::gfp; rol-6(d)], a translational fusion.

syIs63: Is[pBP164 (cog-1prom::gfp; dpy-20(+))], a 5′ fusion [Palmer et al. 2002].

syIs63: Is[cog-1::gfp; dpy-20(+)] [Palmer et al. 2002], a translational fusion.

otEx990: Ex[cog-1prom::gfp; rol-6(d)], a 5′ fusion.

otEx1069–1071: Ex[cog-1prom::gfp], a 5′ fusion.

otEx1006–1007: Ex[pBP159 (cog-1::gfp); rol-6(d)], a translational fusion.

otEx1066–1068: Ex[pBP159 (cog-1::gfp); unc-122::gfp], a translational fusion.

PD8105: Ex[unc-37::gfp; rol-6] [Kelly et al. 1997].

Mutant screen for lsy genes

otIs6 or otIs3 animals were mutagenized with ethyl methane-sulfonate (EMS) using standard procedures [Brenner 1974]. The progeny of individually picked F1 animals derived from the mutagenized P0 population were analyzed under a dissecting scope equipped with a fluorescent light source. Using otIs6 as a marker, we screened through 3200 haploid genomes, and using otIs3, we screened through 12,200 haploid genomes. A total of 28 mutants were retrieved. Through chromosomal linkage and complementation testing, we found these mutants to define 11 complementation groups; one mutant has not yet been assigned to a group. Representatives from each complementation group were backcrossed and transferred into several different gfp transgenic backgrounds that assess gcy-5, gcy-7, and lim-6 expression (otIs6, otIs3, otIs114, ntIs1).
SNP-based mapping

We made use of SNPs present in the Hawaiian *C. elegans* isolate CB4856 identified by the Washington University Genome Sequencing Center and by Ronald Plasterk and colleagues [Wicks et al. 2001]. The respective mutant strain was crossed with CB4856 and resultant mutant F2 progeny were singled out. Their progeny were lysed using standard procedures, genomic fragments that included the SNP were amplified by PCR, and the SNP was analyzed by RFLP. In order to facilitate the identification of recombinants, mutant animals were in some cases marked with a visible marker and mutant recombinants that lost the visible marker were chosen for SNP analysis. *che-1*, *cog-1*, and *lin-49* were identified as *lsy* genes after initial SNP mapping of the *lsy* alleles to chromosomal intervals that contained these genes (*cog-1*, LGII, between K10H10 and Y53F4B, *lin-49*, LGIV, between cosmids D2096 and ZK596) and subsequent complementation testing and allele sequencing.

Positional cloning of *ceh-36(ot79)*

Of all transcription factors described in this paper, *ceh-36* is the only one for which no mutant allele was previously reported. On mapping of the *lsy* mutants retrieved from our mutant screen, we noted that *ot79* was linked tightly to a canonical linkage SNP on the right arm of chromosome X at 16.01 m.u. ([R03E1]; 0/92 recombinants from a cross with CB4856). Left- and right-hand boundaries were established at 15.42 m.u. (C35G3; 1/92 recombinants) and 17.28 m.u. ([F23D12]; 1/92 recombinants), respectively. This region includes 60 predicted protein-coding genes. Because at that point we had shown that numerous transcription factors play a role in asymmetry, we sequenced all four predicted transcription factors in this region (*F28H6.2*, *C37E2.4*, *C37E2.5*, *K04C1.3*) and found a mutation in the coding region of one of them, *ceh-36/C37E2.4*.

DNA constructs

Reporter gene constructs: *gcy-7::rtp* was constructed by replacing *gfp* in the *gcy-7::gfp* construct with dsRed2 ([Clontech]. *gcy-7::gfp* contained 1.3 kb of the 5′ upstream regulatory region of the *gcy-7* gene [Yu et al. 1997]. A *cog-1* transcriptional reporter was constructed by PCR fusion [Hobert 2002] using 4.6 kb of genomic region upstream of the ATG. A *cog-1*-rescuing *gfp* plasmid ([pBP150]) was kindly provided by Takao Inoue and Paul Sternberg [described by Palmer et al. 2002]. A *lin-49* translational *gfp* reporter was constructed by PCR fusion [Hobert 2002], including 2 kb of 5′ region [up to the preceding gene] and all exons and introns of *lin-49*. A *ceh-36* translational *rtp* reporter was constructed by including 5.1 kb of 5′ region and all exons and introns of *ceh-36*, the *rtp* coding region derived from the dsRed2 ([Clontech, Inc.] vector). The marker for all injections was pRF4/rol-6(d) at either 50 or 100 ng/µL. The *unc-37* translational *gfp* reporter was constructed by Kelly et al. [1997].

Heterologous expression constructs: Three promoters were used for heterologous expression, *unc-119* ([Maduro and Pilgrim 1995], *gcy-5*, and *gcy-7* [Yu et al. 1997]. The latter two promoters had to be recombined for *gcy-5*, 3.1 kb of the 5′ upstream regulatory region of the *gcy-5* gene were amplified from N2 genomic DNA using primers containing BamHI sites at either end and subcloned into the *gfp* vector pPD95.75 [a gift from A. Fire] to create *gcy-5::gfp* for *gcy-7*, 1.3 kb of the 5′ upstream regulatory region of the *gcy-7* gene were amplified from N2 genomic DNA using primers with PstI/BamHI sites at either end. The ampli-con was subcloned into the *gfp* vector pPD95.75 to yield *gcy-7::gfp*. A shorter deletion derivative, *gcy-7::HindIII-gfp*, was con-structed by releasing a HindIII fragment from *gcy-7::gfp*, which left 188 bp of the *gcy-7* promoter, which still yielded completely penetrant ASEI expression in adults. The *gcy-5::cog-1* and *gcy-5::unc-37* rescuing plasmids were constructed by replacing *gfp* from *gcy-5::gfp* and replacing it with the *cog-1a* and *unc-37* cDNAs. The *unc-37* cDNA was provided by David Miller [Pillgrad et al. 1997]; the *cog-1a* cDNA was obtained by PCR from an incomplete EST clone, providing the missing 5′ sequence in the primer sequence. The *gcy-7::cog-1* expression plasmid was constructed by replacing *gfp* from *gcy-7::HindIII-gfp* with a *cog-1a* cDNA. *unc-119::lin-6* was constructed by amplifying the genomic *lin-6* region from the start to the stop codon and subcloning it into the pBY103 vector ([Maduro and Pilgrim 1995].

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A transcriptional regulatory cascade that controls left/right asymmetry in chemosensory neurons of *C. elegans*

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