Regulation of chemosensory and GABAergic motor neuron development by the *C. elegans Aristaless/Arx* homolog *alr-1*

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Summary

Mutations in the highly conserved Aristaless-related homeodomain protein ARX have been shown to underlie multiple forms of X-linked mental retardation. *Arx* knockout mice exhibit thinner cerebral cortices because of decreased neural precursor proliferation, and also exhibit defects in the differentiation and migration of GABAergic interneurons. However, the role of ARX in the observed behavioral and developmental abnormalities is unclear. The regulatory functions of individual homeodomain proteins and the networks in which they act are frequently highly conserved across species, although these networks may be deployed in different developmental contexts. In *Drosophila*, *aristaless* mutants exhibit defects in the development of terminal appendages, and Aristaless has been shown to function with the LIM-homeodomain protein LIM1 to regulate leg development. Here, we describe the role of the *Aristaless/Arx* homolog *alr-1* in *C. elegans*. We show that *alr-1* acts in a pathway with the LIM1 ortholog *lin-11* to regulate the development of a subset of chemosensory neurons. Moreover, we demonstrate that the differentiation of a GABAergic motoneuron subtype is affected in *alr-1* mutants, suggesting parallels with ARX functions in vertebrates. Investigating ALR-1 functions in *C. elegans* may yield insights into the role of this important protein in neuronal development and the etiology of mental retardation.

Key words: *C. elegans*, *Aristaless/Arx*, Chemosensory neurons, Motoneurons, GABAergic

Introduction

Homeodomain (HD) proteins play crucial roles in multiple aspects of development and differentiation in all metazoans. Although the functions of individual HD proteins and the genetic pathways in which HD proteins act are often remarkably conserved across phyla (e.g. Gehring and Ikeo, 1999; Montalta-He et al., 2002; Reichert, 2002; Zuber et al., 2003), these pathways are often used in different contexts in different species. For example, the Pax-Six-Eya-Dach regulatory network is used in eye development in both vertebrates and invertebrates, but is also required for muscle development in vertebrates (Heanue et al., 1999; Kawakami et al., 2000). Redeployment of a conserved genetic module for the development of different tissue or cell types may underlie key aspects of speciation and the generation of species-specific characteristics. Studying the functions of HD proteins in multiple model systems can lead to the identification of conserved targets and reveal unexpected insights into their regulatory roles.

The functions of the highly conserved paired-type HD protein Aristaless (AL) was first described in *Drosophila* (Campbell and Tomlinson, 1998; Campbell et al., 1993; Schneitz et al., 1993). In *al* mutants, the development of wings, legs and aristae, terminal antennal appendages required for auditory functions and hygrosensation (Gopfert and Robert, 2002; Manning, 1967; Sayeed and Benzer, 1996) are affected. AL was shown to act in a network with the Bar HD and LIM1 LIM-HD proteins to regulate development of the pretarsus, the

distal-most leg segment (Kojima et al., 2000; Pueyo and Couso, 2004; Pueyo et al., 2000; Tsuji et al., 2000). Vertebrate genomes encode multiple AL homologs that have been classified into three groups based on structural and functional properties, and expression patterns: group I genes are primarily involved in skeletal and craniofacial morphogenesis; group II genes are expressed in the central and peripheral nervous system; and group III genes mediate diverse functions (Meijlink et al., 1999). Recently, the functions of *Arx*, a group II murine and human homolog of *Al*, have been described. In mice, knockout of *Arx* results in animals with thin cerebral cortices, owing to decreased neuroblast proliferation in the neocortical ventricular zone, and defects in the differentiation, proliferation and migration of GABAergic interneurons from the ganglionic eminences to the cortex and olfactory bulbs (Kitamura et al., 2002; Yoshihara et al., 2005). Interestingly, mutations in the human *ARX* gene have been associated with highly pleiotropic developmental and behavioral anomalies. These include X-linked lissencephaly with abnormal genitalia (XLAG), mental retardation, infantile spasms and epilepsy (Bienvenu et al., 2002; Stromme et al., 2002a; Stromme et al., 2002b). In both mice and humans, *Arx* is expressed at high levels in the neocortical ventricular zone and in GABAergic interneurons in the ganglionic eminences (Bienvenu et al., 2002; Colombo et al., 2004; Kitamura et al., 2002; Miura et al., 1997; Poirier et al., 2004). These results suggest that ARX plays an important role in regulating both neuroblast proliferation and differentiation and migration of GABAergic

interneurons. However, the targets of ARX and its precise roles in neuronal development remain to be elucidated. Because, together with Fragile X syndrome, mutations in *Arx* may represent the most common cause of mental retardation in males (Sherr, 2003), it is important that the functions of ARX-related proteins are further investigated.

C. elegans provides an excellent model system in which the functions of conserved proteins can be genetically explored. In particular, the roles of specific proteins in the development and function of the nervous system can be easily investigated. The adult *C. elegans* hermaphrodite contains 302 neurons of which 32 are sensory and 26 are GABAergic motoneurons (McIntire et al., 1993b; Ward et al., 1975; White et al., 1986). Molecules and pathways required for the generation and specification of sensory and motoneuron subtypes have been described (Melkman and Sengupta, 2004; Thor and Thomas, 2002). Genes identified to date encode members of well-conserved transcription factor families, including members of several HD protein families. For example, members of the OTX-type and LIM-HD protein families have been shown to play roles in the development and differentiation of several sensory neuron types in *C. elegans* (Lanjuin and Sengupta, 2004; Melkman and Sengupta, 2004). Vertebrate Otx genes can functionally substitute for *C. elegans* Otx genes, suggesting conservation of protein function across species (Lanjuin et al., 2003). Similarly, the mouse PITX2 HD protein has been shown to functionally substitute for the *C. elegans* UNC-30 PITX-type HD protein in the regulation of expression of the glutamic acid decarboxylase gene and differentiation of GABAergic motoneuron subtypes (Eastman et al., 1999; Jin et al., 1994; Jin et al., 1999; McIntire et al., 1993a; Westmoreland et al., 2001). Characterization of additional molecules required for the development of these neuron types not only allows us to understand the principles underlying the generation of distinct neuronal subtypes in an organism, but also provides an opportunity to explore further the roles of conserved proteins in a well-defined system.

Here, we describe characterization of the *C. elegans* homolog of *Arx/Al*, *alr-1* (*Aristaless/Arx*-related). *alr-1* mutants exhibit defects in the specification of the AWA and ASG chemosensory neurons, and we show that similar to its ortholog in *Drosophila*, ALR-1 acts in a pathway with the LIM1 ortholog LIN-11 to regulate the development of both these neuron types. Intriguingly, we also demonstrate that ALR-1 plays a role in the differentiation of GABAergic motoneurons. In *alr-1* mutants, the VD MN type is mis-specified, leading to a partial adoption of DD motoneuron subtype characteristics. These data indicate that some functions of ARX/Aristaless may be conserved across species, and suggest that studying the role of ALR-1 in neuronal development in *C. elegans* may provide insights into the functions of this important protein in other organisms.

Materials and methods

Strains

Animals were grown using standard methods (Brenner, 1974). A strain carrying integrated copies of the *ops-1::dsRed* transgene (*oyIs47*) was generated as previously described (Satterlee et al., 2001).

Expression of the following stably integrated markers was also examined in *alr-1* mutants (neuron type examined is indicated in parentheses): *gcy-8::gfp* (AFD), *srh-142::dsRed* (ADF), *str-1::gfp* (AWB), *str-2::gfp* (AWC), *sre-1::gfp* (ADL), *sra-6::gfp* (ASH and ASI), *str-3::gfp* (ASI), *daf-7::gfp* (ASI), *odr-1::dsRed* (AWC and AWB), *ttx-3::gfp* (AIY) (Hobert et al., 1997; L'Etoile and Bargmann, 2000; Peckol et al., 2001; Ren et al., 1996; Troemel et al., 1995; Troemel et al., 1997; Troemel et al., 1999; Yu et al., 1997) (E. R. Troemel, PhD thesis, University of California, 1999).

Isolation, mapping and cloning of *alr-1*

oy42 and *oy56* alleles were isolated in EMS mutagenesis screens for altered expression of an integrated *odr-7::gfp* transgene *(kyIs38)* or dye-filling defects respectively. The *ok545* allele was generated by the *C. elegans* Gene Knockout Consortium. Mutants were outcrossed at least four times prior to further analysis. *alr-1(oy42)* was mapped with respect to genetic markers, polymorphisms and deficiencies to LG X. Rescue of tested *alr-1* mutant phenotypes was obtained with sequences amplified from the cosmid R08B4 (nucleotides 13223-19971). The molecular nature of the lesions in the *alr-1* alleles was determined by sequencing.

Expression constructs and generation of transgenic animals

The *alr-1p::alr-1* and *alr-1p::Al* constructs were generated by fusing 2.8 kb of *alr-1* upstream promoter sequences to *Al* or *alr-1* cDNAs (gifts of G. Campbell and Y. Kohara, respectively) in a *C. elegans* expression vector (gift of A. Fire). The *unc-30p::alr-1* construct was generated by fusing 2.5 kb of *unc-30* promoter sequences upstream of an *alr-1* cDNA. *unc-30p::unc-55* was a gift from W. Walthall.

Germline transformations were performed using standard techniques (Mello and Fire, 1995). All transgenes were injected at 30 ng/μl along with one of the following co-injection markers: 100 ng/μl pRF4 *rol-6(su1006)*, 50 ng/μl *unc-122::gfp* (Miyabayashi et al., 1999) or *unc-122::dsRed*.

Cell/lineage autonomy of ALR-1 function

A rescuing *alr-1* genomic fragment was injected along with *osm-6::dsRed* (gift of A. Lanjuin) at 15 ng/μl each into an *alr-1(oy42)* strain containing stably integrated copies of an *odr-10::gfp* fusion gene (*kyIs37*). L1-L3 larvae were incubated in 1:500 dilution of DiO for 1 hour. *osm-6::dsRed*-expressing animals were scored for the presence or absence of *odr-10::gfp* expression, and the corresponding presence or absence of *osm-6::dsRed* expression in ASI or ASK neurons on the same side. Presence of the array scored as a result of perdurance of *osm-6::dsRed* expression is unlikely as we could detect expression in either of the lineally related ASK or ADL neurons on a given side. It is possible that the number of AWA neurons lacking *odr-10::gfp* expression is an overestimation, as *gfp* in the AWA neurons was often difficult to detect in the background of fluorescence because of *dsRed* expression and dye filling.

Behavioral assays

Single animal olfactory assays

Animals with the desired *odr-7::gfp*-expressing phenotype were selected under 400× magnification, and allowed to recover on food for at least 2 hours prior to analysis. Single animals were assayed essentially as described (Bargmann et al., 1993). The behavior was scored as wild type if the animal entered a region of defined diameter surrounding the odorant without entering a similar region around the control diluent during the course of the assay. Statistical significance was determined using a chi-square test.

Dye-filling

Animals were incubated in 1:100 DiI or 1:1000 DiO in M9 for 2 hours, washed twice and let recover on food for at least 30 minutes prior to analysis.

Dauer assays

Dauer assays were performed essentially as previously described (Lanjuin and Sengupta, 2002).

Osmotic avoidance assays

Worms were placed on one half of a 5 cm plate surrounded by a barrier of 4M fructose with a point source of an attractive odorant on the opposite side. The percentage of worms that stayed within the barrier was scored after 30 minutes.

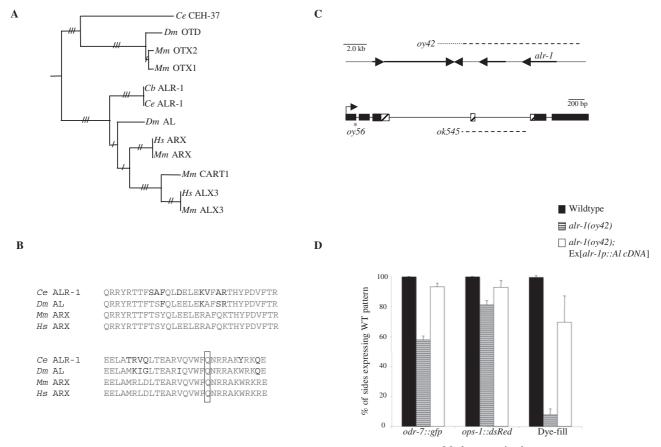### Generation of anti-ALR-1 antibodies and immunocytochemistry

Sequences encoding the C-terminal 182 amino acids of ALR-1 were cloned into the pGEX4T-1 (Pharmacia) vector. The fusion protein was expressed in *E. coli* and purified using Glutathione Sepharose beads. Polyclonal antibodies were generated in rats by Cocalico Biologicals. Sera were affinity purified before use. Staining with anti-ALR-1, anti-ODR-7 and anti-GABA antibodies was performed as described (McIntire et al., 1992; Sarafi-Reinach et al., 2001). Animals were viewed under a Zeiss Axioplan microscope equipped with

epifluorescence and images were captured using a CCD camera (Hamamatsu). Images were analyzed using Openlab (Improvision) and Adobe Photoshop software (Adobe Systems).

Results

alr-1 encodes the *C. elegans* homolog of *Drosophila Aristaless* and vertebrate *Arx*

sns-10(oy42) animals were isolated in a screen for mutants exhibiting altered expression of an *odr-7::gfp* transgene in the AWA olfactory neurons (see below). Genetic mapping and transformation rescue experiments identified *sns-10* as the *R08B4.2* gene. Phylogenetic analyses had previously suggested that *R08B4.2* encodes the *C. elegans* ortholog of *Drosophila* Aristaless, and appears to be most closely related to the vertebrate group II ARX homeoprotein (Galliot et al., 1999) (Fig. 1A). Based on sequence and functional conservation, *sns-10* has been renamed *alr-1*. The homeodomain of ALR-1 is

Fig. 1. *alr-1* encodes the *C. elegans* homolog of Aristaless/ARX. (A) Phylogenetic tree of the homeodomains of Aristaless/ARX family members. The homeodomain sequences were aligned and analyzed using Bonsai 2.1 (http://calliope.gs.washington.edu/software/index.html). The homeodomains of OTD/OTX proteins were used as outliers in this analysis. The frequency at which a particular branch appears in 1000 bootstrap replicates are indicated by hatchmarks: (/) – <50%; (//) – 51-80%; (///) – 81-100%. (B) Alignment of the homeodomains of ALR-1 (*Ce*), AL (*Dm*) and ARX (*Mm, Hs*). Identical residues are indicated in gray. The Q50 residue is boxed. (C) (Top) *alr-1* and neighboring predicted open reading frames present on the cosmid R08B4. The orientation of each ORF is indicated by an arrow. Sequences deleted in *alr-1(oy42)* are indicated by a dashed line; inverted sequences are indicated by a dotted line. (Bottom) Exon/intron structure of *alr-1*. Sequences encoding the homeodomain are hatched. Sequences deleted in *alr-1(ok545)* are indicated by a dashed line. Asterisk indicates a point mutation in the first splice donor site in *alr-1(oy56)*. (D) *Drosophila Al* can functionally substitute for *alr-1* in the regulation of *odr-7::gfp* and *ops-1::dsRed* expression, and for the dye-filling phenotype. The *Al* cDNA was expressed under the *alr-1* promoter. Adult animals grown at 25°C were examined. *n*>100 for each.

Table 1. Mutations in *alr-1* affect gene expression in the AWA and ASG chemosensory neurons

Neuron type	Marker*	Genotype†	% of sides showing expression in neuron number:		
			1	0	2
AWA	*odr-7::gfp*	Wild type	100	0	0
		alr-1(oy42)	57	35	8
		alr-1(oy56)	71	22	7
		alr-1(oy42);Ex[*alr-1 genomic*]	96	3	1
		alr-1(oy56);Ex[*alr-1 genomic*]	98	1	1
		alr-1(oy42);Ex[*alr-1p::alr-1* cDNA]	87	9	4
		alr-1(oy42);Ex[*alr-1p::Al* cDNA]	93	4	3
	ODR-7	Wild type	94	6	0
		alr-1(oy42)	66	28	6
		alr-1(oy56)	81	12	7
		alr-1(ok545)	78	13	9
	odr-10::gfp	Wild type	100	<1	0
		alr-1(oy42)	73	22	5
		alr-1(oy56)	82	10	8
ASG	*ops-1::dsRed*	Wild type	100	0	<1
		alr-1(oy42)	81	19	<1
		alr-1(oy56)	89	11	<1
		alr-1(ok545)	87	13	0
		alr-1(oy42);Ex[*alr-1 genomic*]	98	2	0
		alr-1(oy42);Ex[*alr-1p::alr-1* cDNA]	98	2	0
		alr-1(oy42);Ex[*alr-1p::Al* cDNA]	93	7	0
	Ex[*unc-30::gfp*]	Wild type	76	24	0
		alr-1(oy42)	37	63	0
		alr-1(oy56)	61	39	0

Adult animals grown at 25°C were examined. *n*>100 for each.

*ODR-7 expression was examined by staining with anti-ODR-7 antibodies (Sarafi-Reinach et al., 2001). The *odr-7::gfp* (Sengupta et al., 1994), *odr-10::gfp* (Sengupta et al., 1996) and *ops-1::dsRed* (Sarafi-Reinach et al., 2001) transgenes were stably integrated into the genome. *unc-30::gfp* (Jin et al., 1994) was present on extrachromosomal arrays, and expression from the same arrays was examined in wild-type and mutant backgrounds. Numbers shown are from strains carrying two different *unc-30::gfp* arrays, with the exception of *alr-1(oy56)* in which expression from a single array was examined.

†For extrachromosomal arrays, numbers shown are from at least two independent arrays examined in a given strain background.

~81% and 79% identical to the homeodomains of *Drosophila* AL and human ARX, respectively, and contains a Q50 residue, characteristic of this subclass of Paired-like homeodomains (Galliot et al., 1999; Meijlink et al., 1999) (Fig. 1B). In addition to a conserved homeodomain, members of the AL/ARX family are distinguished by the presence of a C-terminal 16 amino acids 'Aristaless/OAR domain', which is present although not highly conserved in ALR-1 (Meijlink et al., 1999). Expression of the *Al* cDNA driven by the *alr-1* promoter rescued *alr-1(oy42)* mutant phenotypes (Fig. 1D), suggesting conservation of protein function across species.

alr-1(oy42) is a complex deletion-rearrangement that deletes all *alr-1* as well as flanking sequences (Fig. 1C) and is therefore a null allele. There are less severe lesions in two additional *alr-1* alleles (Fig. 1C). *alr-1(oy56)* is a point mutation in the first splice donor site. RT-PCR and sequence analyses indicated that the transcript arising as a consequence of this mutation would be predicted to encode a prematurely truncated protein. However, wild-type transcripts were also detected at low levels, indicating that *alr-1(oy56)* is not a null allele. *alr-1(ok545)* is predicted to encode a protein with a partly deleted homeodomain. All tested defects of *alr-1(oy42)* mutants could be rescued with a genomic fragment containing only *alr-1* sequences (Table 1; Fig. 1D), indicating that these phenotypes were a consequence of loss of *alr-1* functions alone.

ALR-1 regulates gene expression, morphology and functions of the AWA olfactory neurons

In *alr-1* mutants, expression of the *odr-7* nuclear receptor gene was lost in one or infrequently, in both AWA olfactory neurons, as assayed by expression of an *odr-7::gfp* transgene and by staining with anti-ODR-7 antibodies (Fig. 1D; Fig. 2B-C; Table 1). We also observed ectopic expression of *odr-7* in a second sensory neuron at a lower penetrance (Fig. 2D; Table 1). Loss or ectopic expression of *odr-7* was not correlated on the left and right sides of individual animals, enabling us to quantitate the mutant phenotype per side (Table 1). ODR-7 regulates the expression of signal transduction genes, including the *odr-10* olfactory receptor and the *gpa-5* Gα protein genes in the AWA neurons (Jansen et al., 1999; Sengupta et al., 1996). As expected, expression of these genes was similarly affected in *alr-1* mutants (Table 1; data not shown). Gene expression defects were observed as early as the threefold embryonic stage. The AWA neurons exhibited additional defects in *alr-1* mutants. In particular, we observed morphological defects in the dendritic structures, such that the dendrites failed to fully elongate to the anterior amphid organ opening (Fig. 2F). Eighteen and 17% of AWA neurons that could be visualized (*n*>100) exhibited severe dendritic elongation defects in *oy42* and *oy56* animals, respectively. Mutations in *alr-1* did not affect expression of a subset of marker genes in the AWB, AWC, ADL, ASH, ASI, ADF and AFD sensory neurons, and AIY interneurons (data not shown; see Materials and methods). However, 45% of AFD thermosensory neurons and 4% of ADF chemosensory neurons (*n*>65) also exhibited a range of dendritic defects in *alr-1(oy42)* animals.

odr-7 mutants are unable to respond to all AWA-sensed attractive odorants including diacetyl (Sengupta et al., 1994).

We examined the olfactory behaviors of *alr-1(oy42)* and *alr-1(oy56)* mutant animals lacking *odr-7* expression (Fig. 3). Although the overall olfactory responses of *alr-1(oy42)* mutants were diminished, *oy42* and *oy56* mutants expressing *odr-7* in both AWA neurons retained attractive responses to diacetyl. However, animals lacking *odr-7* expression in both AWA neurons showed a strong defect in this response. This defect was specific to the AWA neurons, as responses to the chemicals benzaldehyde and 2,3-pentanedione, mediated by the AWC olfactory neurons (Bargmann et al., 1993), were unaltered upon loss of *odr-7* expression in both AWA neurons (Fig. 3; data not shown).
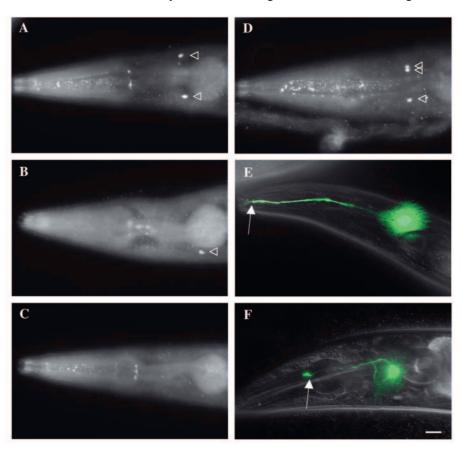
In *odr-7* mutants, the AWA neurons have been shown to misexpress the AWC-specific *str-2* olfactory receptor gene (Sagasti et al., 1999). However, in *alr-1* mutants, *str-2* was not ectopically expressed in the AWA neurons upon loss of *odr-7* expression (data not shown). Additional sensory neuron-specific markers examined (see Materials and methods) were also not ectopically expressed in the AWA neurons in *alr-1* mutants. To determine whether the AWA neurons in *alr-1* mutants were generated, and whether they expressed general sensory neuronal features, we examined expression of an *osm-6::dsRed* fusion gene. *osm-6* is expressed in and required for development of the ciliary structures of all sensory neurons in *C. elegans* (Collet et al., 1998; Perkins et al., 1986). We detected *osm-6::dsRed* expression in AWA neurons (identified by relative position with respect to additional *osm-6*-expressing cells), which failed to express the AWA-specific marker *odr-10::gfp* in *alr-1(oy42)* animals (*n*=12 cells). These results suggest that upon loss of ALR-1 function, the AWA neurons are generated and retain general sensory neuronal characteristics, but fail to adopt neuron type-specific fates.

ALR-1 regulates the differentiation of, and represses an AWA-like fate in, the ASG lineal sibling neurons

The AWA olfactory and the ASG chemosensory neurons arise from the terminal cell division of the ABpl/raapapaa precursors (Sulston et al., 1983). To determine whether mutations in ALR-1 also affect differentiation of the sibling ASG neurons, we examined expression of the markers *ops-1::dsRed* and *unc-30::gfp* in *alr-1* mutants. Expression of both markers in the ASG neurons was affected in *alr-1* mutants (Table 1). Similar to the defects observed in the AWA neurons, marker expression was lost in one or at a lower penetrance, in both ASG neurons. However, we did not detect ectopic expression of either marker. To determine whether gene expression defects in the AWA and ASG sibling neurons were correlated, we examined the expression of both *odr-7::gfp* and *ops-1::dsRed* in individual
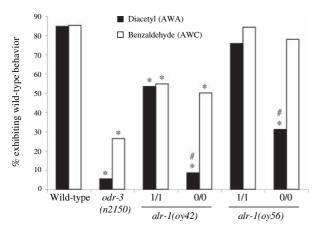

Fig. 2. Mutations in *alr-1* affect gene expression and morphology of the AWA olfactory neurons. (A-D) Top-down views of adult wild type (A), or *alr-1(oy42)* (B-D) animals stained with anti-ODR-7 antibodies. Nuclei of the AWA neurons are indicated by arrowheads. *alr-1(oy42)* mutants exhibit loss of ODR-7 expression in one (B) or both (C) AWA neurons, or ectopic ODR-7 expression (D). (E,F) Lateral view of merged Nomarski and fluorescent images of a wild-type (E) or *alr-1(oy42)* animal (F) expressing an *odr-10::gfp* fusion gene that allows visualization of the AWA sensory dendrite. Arrows indicate dendritic termination points. Scale bar: 10 μm.

alr-1 mutant animals. As shown in Table 2, loss of marker expression in an AWA or ASG neuron was not correlated with loss of expression in its sibling.

Approximately 8% of *alr-1* mutants exhibited ectopic ODR-7 expression in another cell type (Table 1). Although we could not definitively identify the ectopic ODR-7-expressing cells due to some disorganization in the heads of *alr-1* mutants, we investigated the possibility that the sibling ASG neurons were adopting AWA fate. Adoption of AWA fate is expected to be concomitant with loss of ASG-specific gene expression. We noted that although only 10-18% of sides in *alr-1* mutants failed to express *ops-1::dsRed* (Tables 1 and 2), >99% of sides (*n*=56) exhibiting ectopic *odr-7::gfp* expression lacked *ops-1::dsRed* expression (Table 2). Rarely, we also detected colocalization of ectopic *odr-7::gfp* with *ops-1::dsRed* expression. Taken together, these results suggest that a subset of ASG neurons adopt AWA-like characteristics in *alr-1* mutants. However, it is possible that additional cell types may also infrequently adopt an AWA fate. Thus, ALR-1 appears to regulate the differentiation of both the AWA and ASG lineal sibling neurons, and moreover, ALR-1 acts to repress the AWA fate in a subset of ASG neurons.

Fig. 3. *alr-1* mutants fail to respond to an AWA-sensed odorant. All strains except *odr-3(n2150)* contain stably integrated copies of *odr-7::gfp*. The responses of single animals of the indicated genotypes to a point source of a 1:1000 dilution of diacetyl or 1:200 dilution of benzaldehyde were examined. *alr-1(oy42)* and *alr-1(oy56)* animals expressed *odr-7::gfp* in both (1/1) or neither (0/0) AWA neuron(s). Asterisks indicate responses that are significantly different from wild-type responses (*P*<0.001). #, responses of animals lacking *odr-7::gfp* expression in both AWA neurons that are significantly different from the responses of animals of the same genotype expressing *odr-7::gfp* in both AWA neurons at *P*<0.001; *n*≥30 for each.

ALR-1 acts in a parallel pathway with the forkhead domain protein UNC-130 to specify AWA and ASG fates

The forkhead domain-containing protein UNC-130 has previously been shown to regulate asymmetric division of the AWA/ASG precursors (Sarafi-Reinach and Sengupta, 2000). Similar to *alr-1* mutants, the ASG neurons fail to express ASG-like characteristics, and instead adopt an AWA-like fate in *unc-130* mutants. Expression of AWA-specific markers is also lost at a low penetrance in *unc-130* mutants. To determine whether ALR-1 and UNC-130 act in a linear or parallel pathway to regulate AWA and ASG development, we examined AWA- and ASG-specific gene expression in *unc-130; alr-1* double mutants. We observed an almost complete loss of *odr-7* expression in *unc-130(ev505); alr-1(oy42)* and *unc-130(ev505); alr-1(oy56)* double mutants (Table 3). We also observed a significant increase in the penetrance of loss of *ops-1::dsRed* expression in *unc-130(ev505); alr-1(oy42)* double mutants when compared with either single mutant alone (Table 3). These results suggest that ALR-1 and UNC-130 act in parallel to specify AWA and ASG fates.

ALR-1 and the LIM homeodomain protein LIN-11 act in a linear pathway to specify AWA and ASG fates

Previously, we have shown that the *C. elegans* ortholog of the LIM1 LIM homeodomain transcription factor LIN-11 specifies the fates of the AWA and ASG neurons (Sarafi-Reinach et al., 2001). In the AWA neurons, transient expression of *lin-11* during late embryonic/early larval stages is necessary to initiate *odr-7* expression, whereas in the ASG neurons, *lin-11* expression is maintained through all postembryonic stages. This differential temporal regulation of *lin-11* expression is important for correct fate specification, as forced maintenance of *lin-11* expression in the AWA neurons has been shown to result in defects in AWA differentiation (Sarafi-Reinach et al., 2001).

To investigate possible regulatory relationships between ALR-1 and LIN-11, we examined AWA- and ASG-specific gene expression in double mutants. We found that *odr-7* and *ops-1::dsRed* expression was lost to a similar extent in *lin-11(n389); alr-1(oy42)* double mutants as in *lin-11(n389)* mutants alone (Table 3), suggesting that ALR-1 and LIN-11 act in a linear pathway to regulate gene expression in the AWA and ASG neurons. In both neuron types, a higher percentage of *lin-11(n389)* than *alr-1(oy42)* mutants exhibited loss of gene expression, indicating that genes in addition to *alr-1* may play a role in regulating *lin-11*. Alternatively, *lin-11* may function upstream of *alr-1* and additional genes to regulate AWA and ASG fate. To investigate whether ALR-1 acts upstream or downstream of *lin-11*, we examined the expression of a stably integrated *lin-11::gfp* fusion gene in *alr-1* mutants. We noted that ~15% of *alr-1(oy42)* (*n*=87) when compared with 0% of wild-type AWA neurons (*n*=26) exhibited persistent expression of *lin-11::gfp* through late larval stages. We also observed loss of *lin-11::gfp* expression in 40% of ASG neurons of *alr-1(oy42)* mutants (*n*=87). Taken together with the observation that *alr-1* expression was not detected in postmitotic neurons (see below), these results suggest that, ALR-1 acts upstream of *lin-11* in part to downregulate *lin-11* expression in later larval stages in the AWA neurons, whereas ALR-1 promotes *lin-11* expression in the ASG neurons. Interestingly, *Lim1*, the *Drosophila* ortholog of *lin-11* has been shown to be regulated

Table 2. Loss of gene expression in the AWA and ASG neurons is not correlated in *alr-1* mutants

Genotype*	% sides showing expression pattern[†]					Other[‡]
Wild type	100	0	0	0	0	<1
alr-1(oy42)	48	10	33	1	8	<1
alr-1(oy56)	80	2	9	<1	8	1
alr-1(ok545)	76	4	11	<1	9	0

Adult animals grown at 25°C were examined. *n*>320 for each.

*Each strain contains *odr-7::gfp* and *ops-1::dsRed* transgenes stably integrated into the genome except for *alr-1(ok545)* where ODR-7 expression was detected by staining with anti-ODR-7 antibodies.

[†]*odr-7::gfp* expression in an AWA neuron is indicated in green; *ops-1::dsRed* expression in an ASG neuron is indicated in red.

[‡]This category includes rare animals exhibiting ectopic *ops-1::dsRed* expression in a variable number of cells, and animals in which *odr-7::gfp* and *ops-1::dsRed* expression are colocalized.

Table 3. ALR-1 acts in a parallel pathway with the forkhead domain protein UNC-130 and in a linear pathway with the LIM homeodomain protein LIN-11 to regulate AWA and ASG neuronal fate

Genotype	% sides showing *odr-7* expression in number of neurons*			% sides showing *ops-1* expression in number of neurons†		
	1	0	2	1	0	2
Wild type	100	0	0	100/*88*	0/*11*	0/*1*
alr-1(oy42)	55	40	5	89/*71*	11/*29*	*<1*/*<1*
alr-1(oy56)	88	8	4	95	5	<1
unc-130(ev505)	57	11	32	*34*	66	*0*
lin-11(n389)	44	56	0	38/*26*	62/*74*	0/*0*
unc-130(ev505); alr-1(oy42)	1	99	0	*4*	95	*1*
unc-130(ev505);alr-1(oy56)	1	99	0	ND	ND	ND
lin-11(n389); alr-1(oy42)	44	55	1	17	83	0
lin-11(n389); alr-1(oy56)	38	61	1	ND	ND	ND
lin-11(n389); unc-130(ev505)	9	91	<1	*1*	99	*0*

Adult animals grown at 20°C were examined. $n \geq 200$ for each.

*The expression of stably integrated *odr-7::gfp* transgenes were examined in all cases except for *lin-11; unc-130* animals where ODR-7 expression was examined by staining with anti-ODR-7 antibodies.
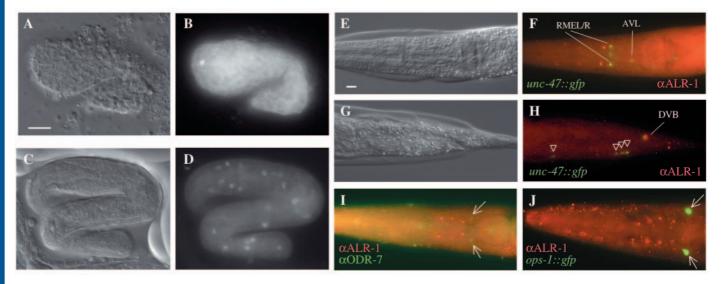
†The expression of *ops-1::dsRed* was examined from stably integrated transgenes or from extrachromosomal arrays (italics). For extrachromosomal arrays, numbers shown are an average from strains carrying two independent arrays. Expression from the same arrays was examined in wild-type, *alr-1, unc-130* and *unc-130; alr-1* double mutants. Expression from different arrays was examined in *lin-11*, *unc-130* and *lin-11; unc-130* double mutants due to the difficulty of mating into the *lin-11* mutant strain.

by AL and acts together with AL to regulate the development of the distal-most compartment of the leg (Pueyo and Couso, 2004; Pueyo et al., 2000; Tsuji et al., 2000).

To further investigate the proposed regulatory relationships among *alr-1*, *lin-11* and *unc-130*, we also examined marker expression in *lin-11; unc-130* double mutants. In both AWA and ASG neurons, we observed a highly penetrant loss of marker expression in *lin-11(n389); unc-130(ev505)* double mutants (Table 3), consistent with the hypothesis that ALR-1 functions in a linear pathway with LIN-11 but in a parallel pathway with UNC-130 to regulate cell fate.

alr-1 is expressed in a spatiotemporally dynamic manner in neuronal and non-neuronal cells

To examine the expression pattern of ALR-1, we raised polyclonal antibodies against the less well-conserved C-terminal sequences of ALR-1. Staining was first evident in 1.5-fold embryos (Fig. 4B) and although the spatial expression was dynamic, stained neuronal and non-neuronal cells were also observed at later embryonic stages. In larvae and adults, ALR-1 expression was observed in multiple neuronal and non-neuronal cells (including epidermal cells) in the head, neuronal cells in the tail and in the GABAergic DD and VD motoneurons

Fig. 4. Expression pattern of *alr-1*. (A-D) Embryonic expression of ALR-1. (A) Nomarski image and (B) anti-ALR-1 antibody staining of a 1.5-fold embryo. ALR-1 expression is observed in two cells in the anterior, only one of which is visible in this plane of focus. (C) Nomarski and (D) anti-ALR-1 antibody stained threefold embryo exhibiting ALR-1 expression in multiple cells in the head, body and tail. Shown is a merge of three different focal planes. (E-J) Post-embryonic expression of ALR-1. Adult animals expressing the GABAergic neuronal marker *unc-47::gfp* in the head (E,F) or tail (G,H) were co-stained with anti-ALR-1 antibodies. Identified cells co-expressing both markers are labeled. Ventral cord motoneurons expressing both markers are indicated by arrowheads. Additional ALR-1-expressing cells were not identified. (I,J) Adult animals stained with anti-ODR-7 antibodies (I) or expressing *ops-1::gfp* (J) were stained with anti-ALR-1 antibodies. ODR-7-expressing AWA neurons and *ops-1::gfp*-expressing ASG neurons are indicated by arrows. No ALR-1 expression was observed in these neurons. Scale bar: 10 μm.
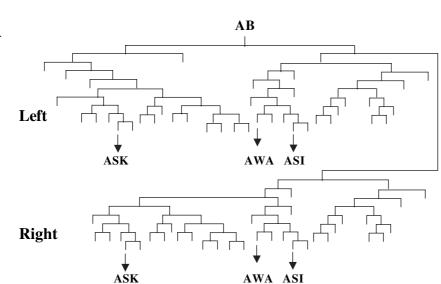
(MNs) in the ventral nerve cord (Fig. 4F,H). Consistent with ALR-1 being a transcription factor, expression was exclusively nuclear in all observed cell types. No staining was observed in *alr-1(oy42)* mutants (data not shown). To determine whether ALR-1 was expressed in the AWA and ASG neurons, we examined colocalization of anti-ALR-1 staining with expression of *odr-7::gfp* and *ops-1::dsRed*, which are expressed postmitotically. However, we did not observe colocalization with these markers in threefold embryos, larvae or adults (Fig. 4I,J; data not shown), although transient expression cannot be ruled out. As ARX has been shown to be expressed in and required for the differentiation of GABAergic neurons in vertebrates (Colombo et al., 2004; Kitamura et al., 2002; Poirier et al., 2004), and ALR-1 is expressed in the GABAergic ventral cord motoneurons, we further investigated whether ALR-1 is expressed in additional GABAergic cells by examining colocalization of anti-ALR-1 staining with *unc-47::gfp* expression. *unc-47* encodes a vesicular GABA transporter and is expressed in all GABAergic neurons (McIntire et al., 1997). Intriguingly, we observed ALR-1 expression in 24 of 26 GABAergic neurons, including the 13 VD and 6 DD, and the RME L/R, AVL, RIS and DVB neurons throughout postembryonic development (Fig. 4F,H; data not shown).

ALR-1 may act in the AWA/ASG lineage to regulate chemosensory neuron development

As ALR-1 is expressed in multiple cell types, it may act cell autonomously or cell nonautonomously to regulate AWA/ASG development. To address this issue, we first attempted to rescue the chemosensory neuronal defects by expressing an *alr-1* cDNA under specific promoters. However, expression of *alr-1* driven by the *unc-130*, *osm-6* or the *unc-30* promoters did not rescue the *alr-1(oy42)* phenotypes (data not shown).

The ASI chemosensory neurons are lineally related to the AWA and ASG neurons such that these three neuron types arise from the common ABp(l/r)aapap precursors via two (AWA and ASG) or three (ASI) additional cell divisions (Fig. 5A) (Sulston et al., 1983). Additional cells arising from these precursors include the AIB interneurons and a cell that undergoes programmed cell death. The ASI neurons can be relatively easily identified via their characteristic cell body positions and via filling with lipophilic dyes (Perkins et al., 1986; White et al., 1986). An additional chemosensory cell type that also fills with dye and is easily identified is the ASK neuron, which arises from a more lineally distant precursor (Fig. 5A) (Sulston et al., 1983). We reasoned that if *alr-1* acts in the AWA/ASG/ASI lineage, presence or absence of *odr-10::gfp* expression in the AWA neurons should correlate more highly with the presence or absence of *alr-1* rescuing sequences in the lineally related ASI neurons than in the ASK neurons. However, if *alr-1* acts elsewhere, then we may detect higher correlation with the presence or absence of *alr-1*-coding sequences in ASK (if *alr-1* acts in this lineage) or similar correlation with expression in both cell types (if *alr-1* acts in a distinct lineage).

To test this hypothesis, we generated *alr-1(oy42)* animals carrying rescuing *alr-1* genomic sequences together with an *osm-6::dsRed* marker gene on an extrachromosomal array. *osm-6::dsRed* is expressed in multiple ciliated sensory neuron types, including the ASK and ASI neurons (Collet et al., 1998) (A. Lanjuin, unpublished). Animals carrying this array are mosaic, as the array is lost randomly at each mitotic division. We first identified transgenic animals which expressed *odr-10::gfp* in an AWA neuron on a side. We then determined whether the array was present in the ASI or ASK neurons on 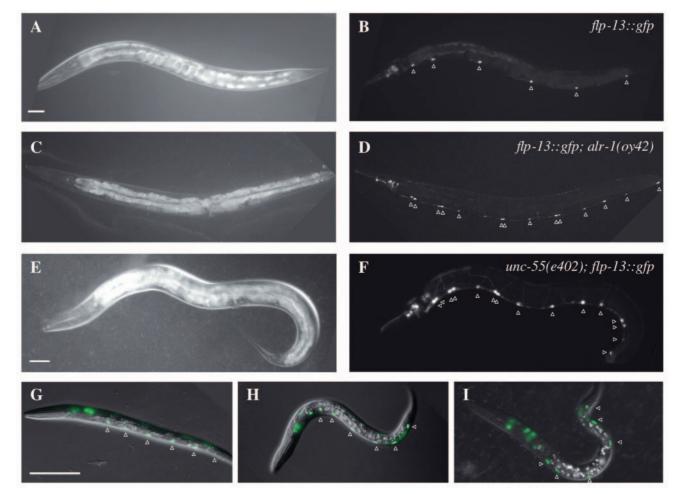that side by examining expression of the *osm-6::dsRed* transgene. Cell identification was further facilitated by dye filling. We found that 82% of *odr-10::gfp*-expressing sides also expressed *dsRed* in the ASI neurons, whereas 59% of sides expressed *dsRed* in the ASK neurons (Fig. 5B). We could not always detect *osm-6::dsRed* expression in the *odr-10::gfp*-expressing AWA neuron because of the relatively weak expression of *dsRed* compared with *gfp*. Conversely, 77% of sides that failed to express *odr-*

Fig. 5. ALR-1 may act in the AWA/ASG lineage to regulate neuronal development. (A) Part of the described embryonic lineage giving rise to the AWA, ASI and ASK neurons on the left and right sides of an animal (Sulston et al., 1983). (B) Presence or absence of *odr-10::gfp* expression in an AWA neuron on a side is more closely correlated with presence or absence of an extrachromosomal array containing *alr-1* rescuing sequences and *osm-6::dsRed* in the ASI than in the ASK neurons in *alr-1(oy42)* animals. * indicates significantly different from ASI at *P*<0.05 by χ-square analysis. *n*=44 each for *odr-10::gfp*+ and *odr-10::gfp*− neurons. L1-L3 larvae grown at 25°C were examined (see Materials and methods).

B

Percentage of *odr-10::gfp*+ sides expressing *osm-6::dsRed* in		Percentage of *odr-10::gfp*- sides not expressing *osm-6::dsRed* in	
ASI	ASK	ASI	ASK
81.8	59.1*	77.3	47.7*

10::gfp also failed to express dsRed in the ASI neurons, when compared with 48% of sides that failed to express in the ASK neurons (Fig. 5B) (see Materials and methods for additional details). These results imply that *alr-1* acts in the AWA/ASG/ASI lineage to regulate development of the AWA and ASG neurons.
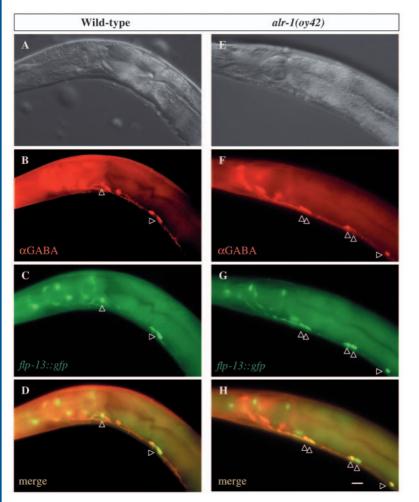
Fig. 6. *alr-1* mutants exhibit ectopic *flp-13::gfp* expression. (A-F) *flp-13::gfp* expression in adult animals. Ventral cord motoneurons expressing *flp-13::gfp* in wild-type (B), *alr-1(oy42)* (D) and *unc-55 (e402)* (F) animals are indicated by arrowheads. Corresponding Nomarski images are shown on the left (A,C,E). (G-I) Merged Nomarski and fluorescent images of *flp-13::gfp* expression in wild-type (G), *alr-1(oy42)* (H) and *unc-55(e402)* (I) L1 larvae. GFP-expressing ventral cord motoneurons are indicated by arrowheads. Scale bar: 50 μm.

Table 4. The VD MNs may partially adopt DD MN characteristics in *alr-1* mutants

Marker	Genotype*	Average number of cells expressing GFP in the VNC (±s.d.)		% animals where all *flp-13::gfp*-expressing cells colocalize with GABA
		L1	Adults	Adults
flp-13::gfp	Wild type	5.9±0.4	6.2±0.6	83
	alr-1(oy42)	6.0±0.1	13.4±2.0	92
	alr-1(oy56)	6.0±0.1	13.1±2.2	ND
	alr-1(ok545)	ND	10.9±2.5	ND
	unc-55(e402)	6.0±0.1	16.1±1.1	88
	Ex*[unc-30::unc-55]*	ND	6.4±0.9	ND
unc-25::gfp	Wild type	ND	18.8±0.6	ND
	alr-1(oy42)	ND	18.8±0.5	ND
	alr-1(oy56)	ND	18.8±0.5	ND
anti-GABA	Wild type[†]	ND	17.9±1.5	–
	alr-1(oy42)[†]	ND	18.2±1	–
	unc-55(e402)[†]	ND	17.4±1.6	–

Animals were grown at 20°C. *n*>75 for strains expressing *flp-13::gfp* or *unc-25::gfp*; *n*>36 for animals stained with anti-GABA antibodies.
*The expression of stably integrated *flp-13::gfp* and *unc-25::gfp* transgenes was examined.
[†]These strains contain integrated copies of *flp-13::gfp*.

Development

Wild-type	alr-1(oy42)

Fig. 7. Ectopic *flp-13::gfp*-expressing cells in *alr-1* mutants are GABAergic. Colocalization (D,H) of *flp-13::gfp* expression (C,G) with anti-GABA staining (B,F) in ventral cord motoneurons of wild-type (A-D) or *alr-1(oy42)* mutants (E-H). Scale bar: 10 μm.

VD motoneurons adopt partial DD motoneuron characteristics in *alr-1* mutants

We next investigated whether the development of GABAergic neurons was affected in *alr-1* mutants. As the development of the 13 VD and 6 DD ventral cord motoneurons has been studied extensively, we focused our attention on these cell types. We observed ectopic expression of the DD MN-specific marker, *flp-13::gfp* (Kim and Li, 2004) in all alleles of *alr-1* mutant animals (Table 4; Fig. 6D). On average, there were 10-13 *flp-13::gfp*-expressing cells in *alr-1* mutant adults when compared with six in wild-type adults. All *flp-13::gfp*-expressing cells were stained with anti-GABA antibodies indicating that the additional cells were GABAergic (Table 4; Fig. 7). However, the total number of GABAergic MNs in the ventral nerve cord were unaltered in *alr-1* mutants as determined by expression of an *unc-25* glutamic acid decarboxylase fusion gene (Jin et al., 1999), and by staining with anti-GABA antibodies (Table 4). These results indicated the possibility that the 13 GABAergic VD MNs were adopting DD MN characteristics in *alr-1* mutants. Unlike the DD MNs, which are generated embryonically, the VD MNs are born postembryonically at the L2 larval stage (Sulston, 1976;
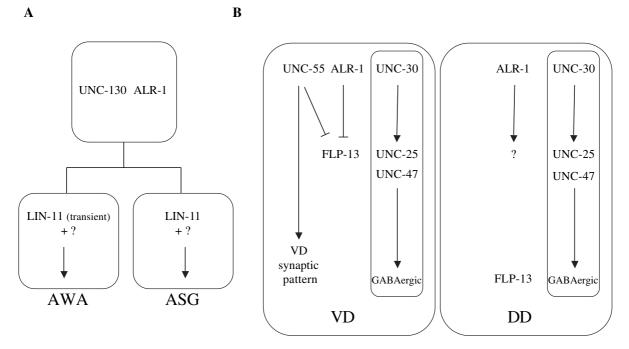
Sulston and Horvitz, 1977; Sulston et al., 1983). Ectopic *flp-13::gfp*-expressing cells in *alr-1* mutants were not observed prior to the L2 larval stage (Table 4, Fig. 6H), further suggesting that the VD MNs were expressing DD MN-specific genes in *alr-1* mutants. Consistent with this hypothesis, markers for other MNs (DA and DB, *unc-129::gfp*; VA and VB, *del-1::gfp*; VC, *ida-1::gfp*) were unaffected in *alr-1* mutants (data not shown).

In adult animals, the VD MNs innervate ventral muscles, whereas the DD MNs innervate dorsal muscles (White et al., 1986). The locations of the VD and DD MN synapses can be visualized by examining localization of a SNB-1 synaptobrevin/VAMP::GFP fusion protein expressed under the *unc-25* promoter (Hallam and Jin, 1998; Jin et al., 1999; Nonet, 1999). In wild-type animals, GFP puncta are observed in the dorsal (DD synapses) and ventral cord (VD synapses) (Hallam and Jin, 1998). We reasoned that if the conversion of VDs into DDs was complete, then in *alr-1(oy42)* mutants, we would observe a dorsal shift in the localization of GFP puncta. However, SNB-1::GFP localization in *alr-1* mutants was indistinguishable from that in wild-type animals, (data not shown), suggesting that VDs may adopt only partial characteristics of the DD MNs. Consistent with this, *alr-1* mutants are not uncoordinated, as might be expected for animals with gross alterations in MN functions or connectivity (McIntire et al., 1993a; McIntire et al., 1993b).

ALR-1 acts in parallel to the UNC-55 COUP-TF-like nuclear hormone receptor to specify VD MN features

The UNC-55 COUP-TF-like nuclear hormone receptor has been shown to be expressed in the VD MNs, where it acts to repress DD MN identity (Walthall and Plunkett, 1995; Zhou and Walthall, 1998). In *unc-55* mutants, the VDs adopt a DD wiring pattern, and this change in innervation pattern is believed to underlie the locomotion defects of *unc-55* mutants (Walthall and Plunkett, 1995; Zhou and Walthall, 1998). In agreement with a VD-to-DD fate transformation in *unc-55* mutants, we observed ectopic *flp-13::gfp* expression in *unc-55(e402)* mutant adults but not in L1 larvae (Fig. 6F,I; Table 4) (Shan et al., 2005). ALR-1 may act downstream or upstream of, or in parallel to UNC-55 to regulate MN differentiation. However, *alr-1* expression in the MNs was unaffected in *unc-55(e402)* mutants (an average of 18±1 ALR-1-expressing cells were observed in *unc-55(e402)* when compared with 18±2 in wild-type animals; *n*>22 each). Similarly, *unc-55::gfp* expression was also unaffected in *alr-1(oy42)* mutants (an average of 14±6 *unc-55::gfp*-expressing cells were observed in *alr-1(oy42)* when compared with 13±5 in wild-type animals; *n*>100 each), suggesting that UNC-55 and ALR-1 may act in parallel to repress *flp-13::gfp* expression in the VD MNs. As ALR-1 is expressed in both the VD and DD MNs, whereas UNC-55 is expressed only in the VD MNs, we next determined whether ectopic expression of UNC-55 in the DD MNs was sufficient to repress *flp-13::gfp* expression. However, the expression pattern of *flp-13::gfp* was unaltered in transgenic

Fig. 8. Model for ALR-1 functions in the AWA/ASG sensory and VD/DD MNs. (A) ALR-1 acts in parallel to UNC-130 and upstream of LIN-11 to specify the AWA and ASG neurons. ALR-1 may act in the precursors or earlier in the lineage. (B) ALR-1 and UNC-55 act in parallel to regulate *flp-13::gfp* expression in the VD MNs. The function of ALR-1 in the DD MNs is unclear. UNC-55 also acts to specify the correct synaptic connectivities of the VD MNs. Expression of the *unc-25* GAD and *unc-47* vesicular GABA transporter genes is regulated by the UNC-30 HD protein (see text for references).

animals expressing *unc-30::unc-55* (Table 4). These results suggest that both UNC-55 and ALR-1 are necessary to repress *flp-13::gfp* expression in the VD MNs, but are not sufficient to repress expression in the DD MNs.

alr-1 mutants exhibit additional pleiotropies

Consistent with *alr-1* expression in multiple neuronal and non-neuronal cell types, *alr-1* mutants exhibit additional phenotypes. A subset of sensory neurons in the amphid and phasmid sensory organs fill with lipophilic dyes such as DiI (Perkins et al., 1986). Developmental or structural defects in either the supporting sheath and socket cells or in the sensory neurons result in dye-filling defects (*dyf* phenotype). Ninety-three and 34% of *alr-1(oy42)* mutant amphids and phasmids, respectively (*n*=224), exhibited dye-filling defects. Consistent with a defect in the supporting non-neuronal cells, dye filling was affected in an all-or-none manner, such that either all amphid neurons on one side of an *alr-1* mutant animal failed to dye fill or all neurons dye filled in the wild-type pattern. *dyf* mutants exhibit additional pleiotropies such as the inability to avoid osmotic shock (Osm phenotype) and failure to enter the alternate dauer developmental stage (Daf-d phenotype) (Starich et al., 1995). *alr-1* mutants are both Osm [~65% of *alr-1(oy42)* and 61% of *alr-1(oy56)* mutants (*n*>100) failed to avoid a high osmolarity solution] and Daf-d [15% *alr-1(oy42)* and 13% of *alr-1(oy56)* animals formed dauers under conditions where 75% of wild-type animals form dauers (*n*>200)]. Taken together with the observation that the differentiation of dye-filling neurons appeared to be unaffected in *alr-1* mutants, these results suggest that the Dyf phenotype may be due to defects in the amphid sheath or socket cells.

These defects may be structural, as the expression of a subset of sheath and socket cell differentiation markers was unaltered in *alr-1* mutants (data not shown). It is unlikely that the AWA and ASG differentiation and/or generation defects are a secondary consequence of the defects in the amphid support cells, as the Dyf phenotype was not correlated with the defects in either gene expression or morphology of the AWA and ASG neurons. In addition, ODR-7 expression was unaltered in *daf-6(e1377)* and *che-14(e1960)* mutants, which exhibit defects in the development of the amphid support cells and additional hypodermal cells (Albert et al., 1981; Michaux et al., 2000) (data not shown). Thus, ALR-1 may affect the differentiation of both a subset of sensory neurons, as well as the supporting non-neuronal cells.

Discussion

Specification of AWA and ASG chemosensory neuron development by ALR-1

A transcription factor cascade involving the UNC-130 forkhead domain protein and the LIN-11 LIM-HD protein specifies the fates of both the AWA and the ASG lineal sisters (Sarafi-Reinach et al., 2001; Sarafi-Reinach and Sengupta, 2000). The strong synergistic effect of mutations in both *unc-130* and *alr-1* on AWA and ASG fate specification suggests that these two proteins act in parallel to regulate AWA and ASG development (Fig. 8A). We have previously suggested that UNC-130 regulates the asymmetric cell division of the AWA/ASG precursors by ensuring segregation of the 'AWA potential' to only the AWA daughter cells (Sarafi-Reinach and Sengupta, 2000). Asymmetric cell division has been

extensively studied in the *Drosophila* nervous system (for reviews, see Betschinger and Knoblich, 2004; Jan and Jan, 2001). Asymmetric localization of intrinsic factors including transcription factors such as Prospero to the neuroblast basal cortex and subsequent inheritance by one of two daughter cells has been shown to be essential for the generation of two distinct daughter cell types. Failure to localize to the cortex or incorrect localization results in the loss of daughter cell types, duplication of sister cell fates and/or defects in daughter cell differentiation. Analogous to *Drosophila* neuroblast cell divisions, we suggest that the defects in AWA and ASG fate specification in *alr-1* and *unc-130* mutants arise as a consequence of improper asymmetric localization and segregation of downstream effector(s), which act combinatorially to regulate AWA and ASG fate. As UNC-130 and ALR-1 act in parallel pathways, they may regulate different sets of effectors perhaps by regulating the transcription of different molecules required to mediate their localization and/or segregation. Alternatively, these proteins may act in parallel pathways to co-regulate a partly shared set of target genes. Interestingly, the penetrance of defects in AWA fate specification is different in *alr-1* and *unc-130* mutants, such that a higher percentage of *unc-130* than *alr-1* mutants exhibit ectopic AWA cells generated at the expense of ASG cells, whereas more *alr-1* than *unc-130* mutants exhibit loss of *odr-7* expression. These phenotypes may arise as a consequence of differential requirements for UNC-130 and ALR-1 in the specification of the AWA and ASG neuron types.

One of the downstream molecules regulated by ALR-1 is *lin-11* (Fig. 8A). Although *lin-11* is required for the differentiation of both the AWA and ASG neurons, the temporal regulation of *lin-11* expression in these neuron types is distinct. In AWA, *lin-11* is expressed transiently, disappearing around the L1 larval stage (Sarafi-Reinach et al., 2001). Loss of *alr-1* disrupts this temporal regulation, allowing expression of *lin-11* to persist to the L4 larval stage in a small number of animals. As the number of animals in which mis-regulation of *lin-11* expression is observed is too low to fully account for the observed loss of *odr-7* expression in *alr-1* mutants, *alr-1* probably plays an additional role in promoting AWA fate. In ASG, ALR-1 acts to promote *lin-11* expression, which is maintained throughout the life of the animal. Thus, a core genetic regulatory network comprising ALR-1, LIN-11 and UNC-130 is used in both the AWA and ASG lineal siblings, but this network functions differently in each of these cells to specify their distinct fates. We note, however, that although the simplest model proposes that ALR-1 acts cell autonomously in the AWA/ASG lineage, it remains possible that ALR-1 acts cell nonautonomously to regulate development of these neuron types.

Role of ALR-1 in GABAergic motoneuron development

alr-1 is expressed in 24 out of 26 GABAergic neurons in *C. elegans*, suggesting a role for this gene in GABAergic neuron development and/or function. Although we did not detect gross abnormalities in the development of additional ALR-1 expressing GABAergic neurons in *alr-1* mutants, we have shown that *alr-1* may play a role in the differentiation of the VD motoneurons. The inhibitory VD (DD) MNs innervate the ventral (dorsal) body muscles and are in turn innervated by

excitatory cholinergic MNs which also innervate dorsal (ventral) body wall muscles (White et al., 1986). Thus, alternate contraction of ventral or dorsal muscles is accompanied by relaxation of dorsal or ventral muscles respectively, resulting in the characteristic sinusoidal locomotory motion (McIntire et al., 1993a; McIntire et al., 1993b; White et al., 1986). The D-type MNs may also regulate wave amplitude (McIntire et al., 1993b).

The GABAergic DD motoneuron marker *flp-13* is ectopically expressed in the VD MNs in *alr-1* mutants, whereas expression of additional GABAergic markers, common to both VD and DD motoneurons is unaffected. In addition, the synaptic connectivities of the VD MNs were also unaltered. We could not further investigate the extent to which the differentiation of the VD MNs was affected in *alr-1* mutants, owing to the lack of additional VD- or DD-specific markers. The *flp-13* gene is predicted to encode at least six FMRFamide-related neuropeptides (Li et al., 1999), two of which have been biochemically isolated from *C. elegans* (Li et al., 1999; Marks et al., 2001). Peptides encoded by *flp-13* have been shown to cause a dramatic inhibition of locomotory behavior and paralysis when injected into *Ascaris suum*, and inhibit pharyngeal activity in *C. elegans* (Marks et al., 2001; Rogers et al., 2001), suggesting that these neuropeptides may act to modulate the inhibitory functions of GABA at the neuromuscular junction. Restriction of *flp-13* expression to the DD MNs in wild-type animals may be important for precise modulation of locomotory behaviors under specific conditions.

The COUP transcription factor UNC-55 has been previously shown to prevent the expression of the DD synaptic pattern in the VD MNs (Walthall and Plunkett, 1995; Zhou and Walthall, 1998). We have shown that similar to ALR-1, UNC-55 also represses expression of *flp-13::gfp* in the VD MNs, although, unlike UNC-55, ALR-1 does not affect the synaptic pattern of VD motoneurons. ALR-1 is not sufficient to repress *flp-13* expression in the absence of UNC-55 function and vice versa, suggesting that functions of both proteins are necessary for repression of *flp-13* expression in the VD MNs. Thus, ALR-1 acts together with a member of the well-conserved COUP transcription factor family to regulate the differentiation of a specific GABAergic MN subtype (Fig. 8B). However, mutations in *unc-55* do not affect AWA development, and mutations in *unc-130* and *lin-11* do not alter *flp-13::gfp* expression (T.M. and P.S., unpublished), suggesting that ALR-1 functions in different pathways to regulate chemosensory and motoneuron development.

Implications for ARX function in vertebrates

Our results indicate that ALR-1 acts in distinct transcriptional cascades to regulate asymmetric cell division of a neuronal precursor and to specify the characteristics of a GABAergic MN subtype in *C. elegans* (Fig. 8). These processes have parallels to the processes regulated by ARX in vertebrates. In *arx* mutant mice, neuroblast proliferation in the cerebral cortex is decreased (Kitamura et al., 2002). Neuroblast proliferation in the ventricular zone occurs via temporally regulated symmetric and asymmetric cell divisions that generate additional neuronal precursors and postmitotic neurons (McConnell, 1995). We speculate that ARX may regulate these cell divisions perhaps by regulating the localization or segregation of determinants such as Numb or Notch (Petersen

et al., 2002; Shen et al., 2002; Wakamatsu et al., 1999; Zhong et al., 1996; Zhong et al., 2000; Zhong et al., 1997). ALR-1 acts in part by temporally restricting expression of *lin-11* in the AWA neurons, and by promoting *lin-11* expression in the ASG neurons. Interestingly, expression of the LIM homeobox genes *Lhx6* and *Lhx9* is abolished in the neocortex and thalamic eminence, respectively, in *Arx* mutant mice, whereas the domain of *Lhx6* expression in the ganglionic eminences is enlarged (Kitamura et al., 2002). Taken together with the observation that *lim1* and *al* function in a network to regulate *Drosophila* leg development (Pueyo and Couso, 2004; Pueyo et al., 2000; Tsuji et al., 2000), these findings suggest that regulatory mechanisms between ARX proteins and LIM-HD proteins may be conserved across species.

ALR-1 acts together with the UNC-55 COUP transcription factor to regulate the differentiation of a GABAergic MN type in *C. elegans*. A COUP-TF protein and the PRDL-B Aristaless/ARX homolog have been shown to act in a network to regulate neurogenesis in Hydra (Gauchat et al., 2004). In vertebrates, COUP transcription factors have been implicated in neurogenesis, neuronal differentiation, migration and axonal guidance (Qiu et al., 1997; Tripodi et al., 2004; Zhou et al., 1999; Zhou et al., 2001). Interestingly, COUP-TFI and COUP-TFII exhibit overlapping spatiotemporal expression patterns with ARX in the developing neocortex, as well as in the lateral and medial ganglionic eminences, which give rise to GABAergic interneurons (Jonk et al., 1994; Liu et al., 2000; Qiu et al., 1994). Moreover, COUP-TFI is co-expressed with the GABAergic neuron marker calbindin in the cortex (Tripodi et al., 2004). These findings suggest the intriguing possibility that COUP and ARX function together to regulate neuronal, and in particular GABAergic, neuronal development. Our results suggest that ARX proteins function in partly conserved genetic networks to regulate the development of different tissue and cell types in different species, and raise the possibility that identification of potential interactors and targets of ALR-1 in *C. elegans* may aid in elucidating ARX function in brain development in vertebrates.

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