The AHR-1 aryl hydrocarbon receptor and its co-factor the AHA-1 aryl hydrocarbon receptor nuclear translocator specify GABAergic neuron cell fate in *C. elegans*

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Summary

The aryl hydrocarbon receptors (AHR) are bHLH-PAS domain containing transcription factors. In mammals, they mediate responses to environmental toxins such as 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Such functions of AHRs require a cofactor, the aryl hydrocarbon receptor nuclear translocator (ARNT), and the cytoplasmic chaperonins HSP90 and XAP2. AHR homologs have been identified throughout the animal kingdom. We report here that the *C. elegans* orthologs of AHR and ARNT, *ahr-1* and *aha-1*, regulate GABAergic motor neuron fate specification. Four *C. elegans* neurons known as RMED, RMEV, RMEL and RMER express the neurotransmitter GABA and control head muscle movements. *ahr-1* is expressed in RMEL and RMER neurons. Loss of function in *ahr-1* causes RMEL and RMER neurons to adopt a RMED/RMEV-like fate, whereas the ectopic expression of *ahr-1* in RMED and RMEV neurons can transform them into RMEL/RMER-like neurons. This function of *ahr-1* requires *aha-1*, but not *daf-21/hsp90*. Our results demonstrate that *C. elegans ahr-1* functions as a cell-type specific determinant. This study further supports the notion that the ancestral role of the AHR proteins is in regulating cellular differentiation in animal development.

Supplemental data available online

Key words: Aryl hydrocarbon receptor (AHR-1), ARNT (AHA-1), HSP90, Neuron, GABA, Cell fate, *C. elegans*

Introduction

The aryl hydrocarbon receptors (AHR) were first identified by their high affinity binding to TCDD or dioxin, the persistent environmental contaminant (Poland et al., 1976). The protein sequences of AHRs are evolutionarily conserved, and contain a basic Helix-Loop-Helix (bHLH) domain at the N-terminal and a PAS (Per/ARNT/Sim) domain (the PAS domain can be further divided into PAS A and PAS B subdomains) in the middle of the protein (reviewed by Hahn, 2002). The bHLH domain binds DNA, and the PAS domain mediates protein-protein interactions and toxin binding (reviewed by Schmidt and Bradfield, 1996). In mammals, the AHRs mediate many of the diverse biochemical, biological and toxicological responses following exposure to TCDD and related compounds (Fernandez-Salguero et al., 1995; Schmidt et al., 1996) (for a review, see Gonzalez and Fernandez-Salguero, 1998). They normally reside in the cytoplasm in a complex with the chaperonins HSP90 (Heat Shock Protein 90) and XAP2/AIP (X-associated protein 2/AhR-interacting protein) (Hahn, 2002). Upon toxin binding, the cytoplasmic AHRs translocate to the nucleus, where they dissociate from HSP90 and XAP2, and form a heterodimer complex with a bHLH-PAS protein, the AHR nuclear translocator (ARNT) (Reyes et al., 1992; Probst et al., 1993; Hahn, 2002). The AHR and ARNT heterodimers bind to specific DNA sequences known as Xenobiotic Response Elements (XREs), and can recruit multiple co-activators to regulate gene expression (Kobayashi et al., 1997; Kumar and Perdew, 1999; Nguyen et al., 1999; Tohkin et al., 2000).

Invertebrates generally do not have a dioxin-induced toxic response, and none of the invertebrate AHRs tested bind dioxin, leading to the hypothesis that the ancestral role of the AHR family is not in toxin response (Hahn et al., 1997; Hahn, 2002). Studies in *Drosophila* have supported this idea. Mutations in the *Drosophila* AHR *spineless* cause reduced sensory bristles and transformation of distal antenna into distal leg (Duncan et al., 1998). This function of Spineless requires Tango, the *Drosophila* ARNT (Emmons et al., 1999). Spineless and Tango bind directly and exhibit genetic interactions. Both Spineless and Tango are normally localized in the nucleus, and, interestingly, the nuclear localization of Tango depends on Spineless.

ahr-1 and *aha-1* are the *C. elegans* homologs of AHR and ARNT, respectively (Powell-Coffman et al., 1998). AHR-1 and AHA-1 bind each other in vitro and can bind XREs in a sequence specific manner in vitro. AHR-1 can also bind rabbit HSP90, but not mammalian XAP2 (Powell-Coffman et al., 1998; Bell and Poland, 2000). A single ortholog of HSP90 in *C. elegans* is encoded by the *daf-21* gene (Birnby et al., 2000).

The null mutation of *daf-21* is early larval lethal, and an unusual mutation of *daf-21(p673)* causes constitutive formation of dauer larvae.

The *C. elegans* nervous system is composed of 302 neurons (White et al., 1986), 26 of which use γ-amino butyric acid (GABA) as the neurotransmitter and regulate body movement, defecation and foraging behaviors (McIntire et al., 1993). These GABAergic neurons fall into at least five types based on morphology and function, including the 19 type D ventral cord motor neurons, RIS, AVL, DVB and four RME neurons. The UNC-30 homeodomain protein controls the specification of the type D ventral cord neurons (Jin et al., 1994). The LIM-6 homeodomain protein regulates subsets of the differentiated aspects of RIS, AVL and DVB neurons (Hobert et al., 1999). The four RME neurons innervate head muscles to control foraging behavior (White et al., 1986; McIntire et al., 1993). Although sharing similar neurotransmitter specificity and the same synaptic targets, the RME neurons can be further divided into two subgroups, based on cell lineage and gene expression. For example, RMEL and RMER are lineally related, and express *lim-6* and the AMPA-type glutamate receptor *glr-1* (Hart et al., 1995). RMED and RMEV do not express these markers; instead they express the ivermectin receptor *avr-15* (Dent et al., 1997). We found that loss of function in *ahr-1* transforms RMEL/RMER neurons into RMED/RMEV-like cells. Ectopic expression of AHR-1 in RMED/RMEV can transform them to an RMEL/RMER-like fate. We provide additional evidence that supports an evolutionarily conserved partnership of AHR and ARNT. Our findings are consistent with the notion that an ancestral role of AHR proteins is in regulating cellular development.

Materials and methods

Strains and genetics

C. elegans were grown on NGM plates at 22.5°C under standard conditions, as described by Brenner (Brenner, 1974). Genetic nomenclature follows the standard *C. elegans* system (Horvitz et al., 1979). The GFP reporter strains used are listed below: *juIs76* ($P_{unc\text{-}25}$GFP) (Huang et al., 2002), *juIs1* ($P_{unc\text{-}25}$SNB-1-GFP) (Zhen and Jin, 1999), *oxIs12* ($P_{unc\text{-}47fl}$GFP) (McIntire et al., 1997), *otIs37* ($P_{unc\text{-}47s}$GFP) (Aurelio et al., 2003), *nuIs25* ($P_{glr\text{-}1}$GFP) (Hart et al., 1995), *otEx406* ($P_{lim\text{-}6r}$GFP) (Hobert et al., 1999), *juEx517* ($P_{avr\text{-}15}$GFP) (Dent et al., 1997) (this study), *vbEx1* ($P_{clh\text{-}6}$GFP) (Bianchi et al., 2001), *iaIs2* (AHR-1::GFP) (Qin, H and J.A.P.-C., unpublished). ZG69 *aha-1(ia1); iaEx28* was used as the starting strain to examine the role of *aha-1* in RME neurons. *aha-1(ia1)* is a deletion mutation and the mutant animals die as first stage larvae. This larval lethality is due to the functional requirement of *aha-1* in pharynx. The *iaEx28* transgene expresses a construct, pHJ32, that has the complete *aha-1* genomic coding sequences driven by a 2.5 kb promoter of the C15C8.2 gene, which is active in non-neuronal cells of the pharynx, and the *iaEx28* transgene expression restores viability to *aha-1(ia1)* homozygous animals. Immunostaining with an AHA-1-specific monoclonal antibody (Jiang et al., 2001) confirms that *aha-1; iaEx28* animals express AHA-1 in the pharynx, but not in neurons in the head or in other cells in the body (H. Jiang, S. Wu and J.A.P.-C., unpublished).

Isolation and mapping of *ju145*

juIs76($P_{unc\text{-}25}$GFP) animals were mutagenized with ethyl methanesulfonate (EMS) following the standard procedure as described (Sulston and Hodgkin, 1988). The cell morphology of RME neurons of mutagenized F2 progeny was examined under a Nomarski fluorescence microscope, and mutant animals were recovered to produce progeny. A total of ~6,200 mutagenized haploid genomes were screened. The *ju145* mutation was outcrossed multiple times before further analysis. Homozygous *ju145* mutant animals exhibited no discernable abnormalities in locomotion, egg laying, or male mating. The foraging behaviors of *ahr-1* animals were observed in parallel with N2, *juIs76*, *glr-1(n2461)*, *unc-25(e156)* and *unc-47(e542)* animals on matched L4 and young adult animals, and the genotypes were blinded. *ju145* was mapped to chromosome I because of its linkage to *dpy-5*. It was further mapped to the *unc-29 dpy-24* interval, near the *hP6* marker, based on the following data: from *ju145/unc-29 hP6 dpy-24* heterozygous animals, 8 out of 37 Unc non-Dpy recombinants, and 5 out of 8 Dpy non-Unc animals, segregated *ju145*; 0 out of 8 Unc *ju145* non-Dpy, and 0 out of 5 Dpy *ju145* non-Unc, segregated *hP6*. *ju145* was uncovered by the deficiencies *qDf7*, *qDf9* and *mnDf111*, but not by *dxDf1*, *dxDf2*, *eDf3*, *nDf23*, *nDf29* or *qDf5*.

Molecular biology

Cosmids were obtained from the Sanger Centre, Hinxton, UK. DNA preparation and subcloning followed standard procedures (Sambrook et al., 1989). The *ahr-1* minimal rescuing construct (pCZ466) was generated by cloning a 10 kb *Apa*I-*Spe*I genomic fragment into pSL1190. To make the pHT101 P*ahr-1*GFP plasmid, a 5377 bp *Hin*dIII-*Bam*HI genomic fragment, which includes over 3 kb of sequence 5′ to the translational start codon, exon1, intron 1 and part of exon 2, was ligated into the corresponding sites of the pPD95.67 GFP expression vector (a gift of A. Fire). To generate $P_{unc\text{-}25}$AHR-1, the *ahr-1* promoter was replaced by the *unc-25* promoter at the *Bst*BI site, which is located 14 bp 5′ to the start ATG codon of C41G7.5/AHR-1. Germline transformation was performed following standard procedures (Mello et al., 1991). The co-injection marker was the dominant pRF4 *rol-6(su1006)* injected at 20 ng/μl. Over 10 transgenic lines expressing $P_{unc\text{-}25}$AHR-1 were obtained, and all showed similar effects. *juEx467*($P_{unc\text{-}25}$AHR-1) was chosen for further analysis.

To identify the lesions in *ju145*, the genomic DNA, including all exons and exon-intron junctions, was amplified from mutant and wild-type animals. DNA sequences were determined using ^{33}P-labeled primers and the *fmol* sequencing kit (Promega), and were confirmed on both strands and from DNA prepared in independent PCRs.

GABA antibody staining and GFP phenotypic analysis

Anti-GABA staining was performed using the glutaraldehyde-paraformaldehyde fixation procedure described by McIntire et al. (McIntire et al., 1992). GFP reporter expression was directly observed under a 63× objective of a Zeiss Axioskop fluorescence microscope equipped with a HQ-FITC filter (Chroma). Images were captured using an AxioCam camera (Zeiss) and analysed using the Axiovision software (Zeiss).

Results

Isolation of *ahr-1(ju145)*, a mutation in the *C. elegans* aryl hydrocarbon receptor

The four RME motor neurons are located at the nerve ring and innervate the head muscles. The positions of their cell bodies show a fourfold symmetry with respect to the nerve ring (Fig. 1A): dorsal (RMED), ventral (RMEV), left lateral (RMEL) and right lateral (RMER) (White et al., 1986). RMEL and RMER are lineally related, and RMED and RMEV come from two separate lineages (Sulston et al., 1983). Each RME cell sends out two processes circling around the nerve ring, with the distal ends of these processes forming synapses to head muscles.

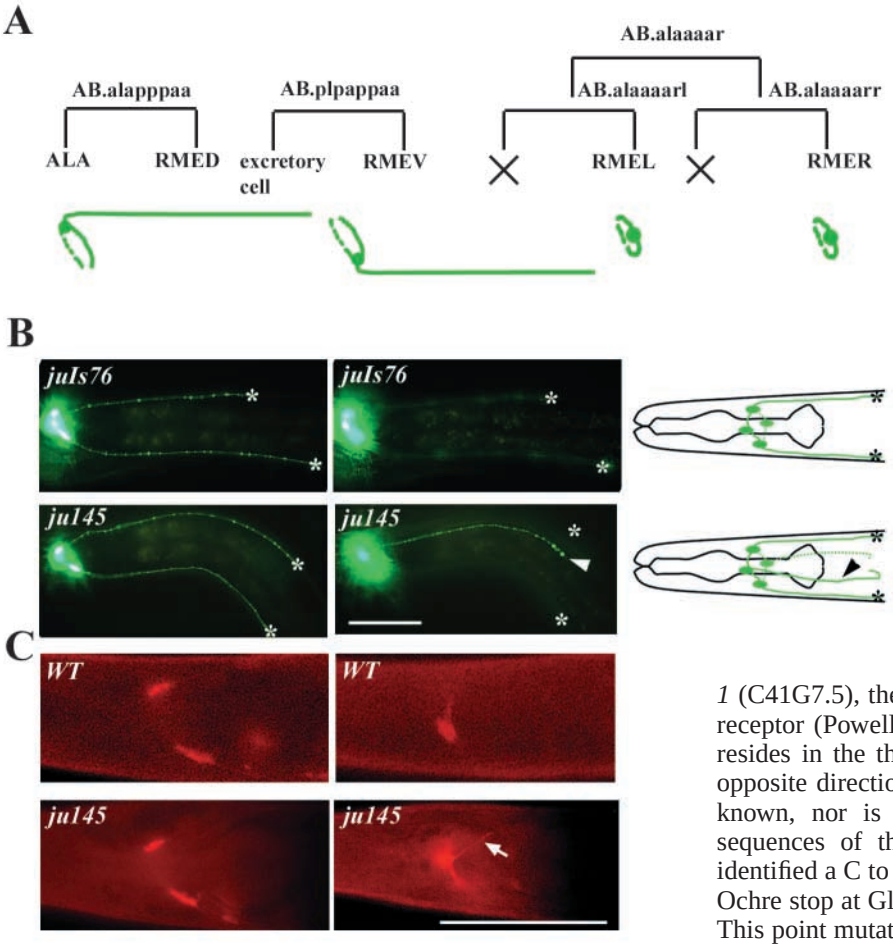

Fig. 1. GABAergic RME neurons. (A) The cell lineage and morphology of RME cells. X, dying cells. (B) $P_{unc\text{-}25}$GFP expression. For wild type (labeled as *juIs76*) and *ju145*, two focal planes are shown, one on RMED/V, the other on RMEL. Schematic illustrations are shown on the right. Asterisks denote the position of the processes from the RMED and RMEV neurons; arrowheads point to the ectopic process from RMEL in *ju145*. (C). Anti-GABA staining reveals defects in *ahr-1* identical to those shown in B. Two focal planes are shown, one on RMED/V, the other on RMEL. Arrow points to the ectopic process from RMEL in *ju145*. Scale bar: 50 μm.

RMED and RMEV have additional processes extending along the dorsal and ventral cords, respectively, and the function of these processes is not known.

We used a GFP transgene driven by the *unc-25* promoter to visualize the RME neurons [the genotype of the marker is *juIs76[*$P_{unc\text{-}25}$*GFP]* (Huang et al., 2002)] (Fig. 1B). *unc-25* encodes glutamic acid decarboxylase/GAD (Jin et al., 1999). In a visual screen for abnormal expression patterns of *juIs76[*$P_{unc\text{-}25}$*GFP]*, we isolated the *ju145* mutation because the mutant animals showed specific defects in the RMEL and RMER, but not RMED and RMEV, neurons (Table 1; and see below).

Table 1. RME neuron defects in *ahr-1* and other mutants

	Percentage of neurons that have abnormal morphology				
	RMED	RMEV	RMEL	RMER	*n*
Wild type	0	0	0	0	>100
ahr-1(ju145)	0	0	100	100	>100
aha-1(ia1)	0	0	100	100	30
*daf-21(p673)**	0	0	0	0	28
$P_{unc\text{-}25}$AHR-1*(juEx467)*	100	100	0	0	>100
ahr-1(ju145); juEx467	100	100	0	0	50
aha-1(ia1); juEx467	0	0	100	100	35
daf-21(p673); juEx467	100	100	0	0	40

**daf-21(nr2081)* has a similar result.

We mapped *ju145* to chromosome I between *unc-29* and *dpy-24*, close to a polymorphism *hP6* (Fig. 2A; see Materials and methods), and rescued the RMEL/R cell defects with transgenes containing the cosmid R30. A 10 kb rescuing DNA fragment contains two predicted genes: *ahr-1* (C41G7.5), the *C. elegans* ortholog of the aryl hydrocarbon receptor (Powell-Coffman et al., 1998), and C41G7.6, which resides in the third intron of *ahr-1* and is transcribed in the opposite direction (Fig. 2B). The function of C41G7.6 is not known, nor is its expression pattern. We determined the sequences of the genomic DNA from *ju145* animals and identified a C to T nucleotide transition that would result in an Ochre stop at Gln302 in the PAS domain of AHR-1 (Fig. 2C). This point mutation is located 2 kb upstream of C41G7.6, and is unlikely to affect the expression of C41G7.6. As shown below, we were able to rescue the RME neuron defects in *ju145* animals by expressing *ahr-1* specifically in RMEL/R neurons. The RMEL/R defects remained the same in *ju145/Df* animals (see Materials and methods). These results are consistent with the conclusion that the RMEL/R neuron defects of *ju145* animals are due to the complete loss of function of *ahr-1*.

RMEL and RMER neurons adopt an RMED/V-like fate in *ahr-1* mutants

In *ahr-1(ju145)* animals the expression level of $P_{unc\text{-}25}$GFP was normal in all four RME neurons. RMED and RMEV had normal cell morphology (Fig. 1B). However, RMEL and RMER exhibited abnormal cell morphology such that, in addition to their circular nerve processes, each extended an ectopic process of variable length growing along the lateral side of the body (Fig. 1B, Table 1). To confirm that this morphological defect is not an artefact of the expression of $P_{unc\text{-}25}$GFP transgene, we first examined the expression of a *unc-47* GFP reporter gene $P_{unc\text{-}47fl}$GFP. *unc-47* encodes a GABA transporter and is normally co-expressed with *unc-25/GAD* in the GABAergic neurons (McIntire et al., 1997; Eastman et al., 1999). We found that in *ahr-1(ju145)* animals the expression of a transgene $P_{unc\text{-}47fl}$GFP that contains the full-length *unc-47* promoter was normal in four RME neurons, and RMEL/R exhibited abnormal cell morphology as seen with $P_{unc\text{-}25}$GFP (n>100, data not shown). We then performed immunocytochemical staining using anti-GABA antibodies to assess the expression of GABA and the cell morphology of

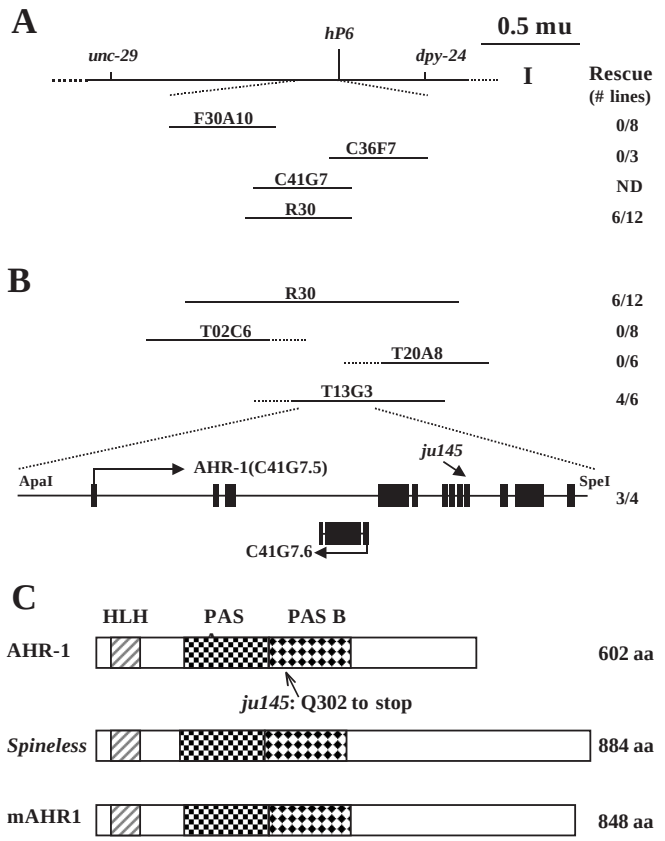

Fig. 2. *ju145* is a mutation in C41G7.5/AHR-1. (A,B) The genetic map position and transformation rescue of *ahr-1(ju145)*. The rescuing activities are shown as number of rescued line/total number of lines. ND, not determined. (C) AHR-1 domain structure and the molecular lesion of *ju145*. HLH, helix-loop-helix; PAS, domain in Per, ARNT and Sim. The PAS domain can be further divided to PAS A and PAS B subdomains.

RME neurons. We found that in all four RME neurons in *ahr-1(ju145)* animals GABA expression was indistinguishable from wild type, and the RMEL/R neurons showed morphological defects identical to those seen with $P_{unc\text{-}47fl}$GFP and $P_{unc\text{-}25}$GFP (Fig. 1C; n>20). To further explore how other differentiated aspects of the RME neurons are affected, we examined the synaptic connections of these neurons using a synaptic GFP marker *juIs1[*$P_{unc\text{-}25}$*SNB-1-GFP]* (Zhen and Jin, 1999). In wild-type animals, RMEL/R and RMED/V neurons make numerous synapses onto head muscles at the distal ends of their ring processes (Fig. 3A). We found that in *ahr-1(ju145)*, fewer synapses were made between RMEL/R and head muscles (Fig. 3B), whereas the synapses between RMED/V and head muscles appeared indistinguishable from wild type (data not shown). These observations indicate that the *ahr-1* mutation specifically affects cellular differentiation of RMEL and RMER neurons. Despite these defects in RMEL/R neurons, the foraging behavior of *ahr-1(ju145)* animals is largely normal by double-blind tests, suggesting that RMEL/R neurons in *ahr-1* mutants provide sufficient synaptic outputs for head muscle movement.

The altered cell morphology of RMEL/R in *ahr-1(ju145)* resembles that of RMED/V cells, suggesting that RMEL/R neurons may be transformed into RMED/V. To further address this interpretation, we examined the expression of several GFP markers that are differentially expressed in RMEL/R and RMED/V neurons. GLR-1, an AMPA-type glutamate receptor, is expressed in RMEL, RMER and other neurons, but not in RMED and RMEV (Hart et al., 1995) (Fig. 3C). In *ju145* animals, RMEL/R cells failed to express $P_{glr\text{-}1}$GFP, whereas $P_{glr\text{-}1}$GFP expression in other neurons was normal (Fig. 3D). LIM-6, a homeodomain protein, is normally expressed in RMEL/R but not in RMED/V cells (Hobert et al., 1999). We found that the expression of a transgene, $P_{lim\text{-}6r}$GFP, in RMEL/R was abolished in *ju145* animals (Fig. 3E,F). It has been reported that in *lim-6* mutants $P_{glr\text{-}1}$GFP expression is normal and RMEL/R neurons are normal (Hobert et al., 1999), indicating that the effect on $P_{glr\text{-}1}$GFP in *ahr-1(ju145)* animals is independent of *lim-6*. In addition, we found that the expression of a transgene $P_{unc\text{-}47s}$GFP that contains a shortened *unc-47* promoter, and which is expressed specifically in RMEL/R but not in RMED/V (Aurelio et al., 2003), was abolished in RMEL/R neurons of *ahr-1(ju145)* animals (Fig. 3G,H). Similarly, the expression of a CIC chloride-channel gene, $P_{clh\text{-}6}$GFP, in RMEL/R neurons was also abolished in *ahr-1(ju145)* animals (see Fig. S1 at http://dev.biologists.org/supplemental/). By contrast, when we examined the expression of $P_{avr\text{-}15}$GFP, which is normally expressed in RMED/V, but not RMEL/R, neurons (Dent et al., 1997) (Fig. 3I), we found that the $P_{avr\text{-}15}$GFP marker was now expressed in RMEL/R cells in *ju145* animals (Fig. 3J-L; Table 2). Taken together, our results support the conclusion that the *ahr-1(ju145)* mutation alters multiple differentiated features of RMEL/R and transforms them into RMED/V-like neurons.

Table 2. RMEL/R adopt RMED/V fate in *ahr-1* mutants

	Percentage of animals that express GFP				
	RMED	RMEV	RMEL	RMER	n
$P_{glr\text{-}1}$GFP	0	0	100	100	50
ahr-1; $P_{glr\text{-}1}$GFP	0	0	0	0	45
$P_{lim\text{-}6}$GFP	0	0	98	100	50
ahr-1; $P_{lim\text{-}6}$GFP	0	0	0	0	34
juEx467; $P_{lim\text{-}6}$GFP	88	90	100	100	66
$P_{unc\text{-}47s}$GFP	0	0	100	100	50
ahr-1; $P_{unc\text{-}47s}$GFP	0	0	0	0	60
$P_{avr\text{-}15}$GFP	100	100	2	0	50
ahr-1; $P_{avr\text{-}15}$GFP	100	100	98	100	38

RMEL and RMER neurons express *ahr-1*, and the ectopic expression of *ahr-1* in RMED/V transforms them into RMEL/R like cells

To understand how *ahr-1* functions, we examined its expression pattern using a GFP reporter gene (Chalfie et al., 1994). A 5.3 kb *ahr-1* genomic fragment that includes over 3 kb of upstream sequences and the first two exons was used to drive GFP expression in transgenic animals. This transgene was expressed in many neurons and the expression pattern was largely identical to a functional AHR-1::GFP [the *ahr-1* expression pattern is currently being investigated in more detail (H. Qin and J.A.P.-C., unpublished)]. We found that the P*ahr-1* GFP was expressed in RMEL and RMER, but not in RMED and RMEV, cells (Fig. 4).

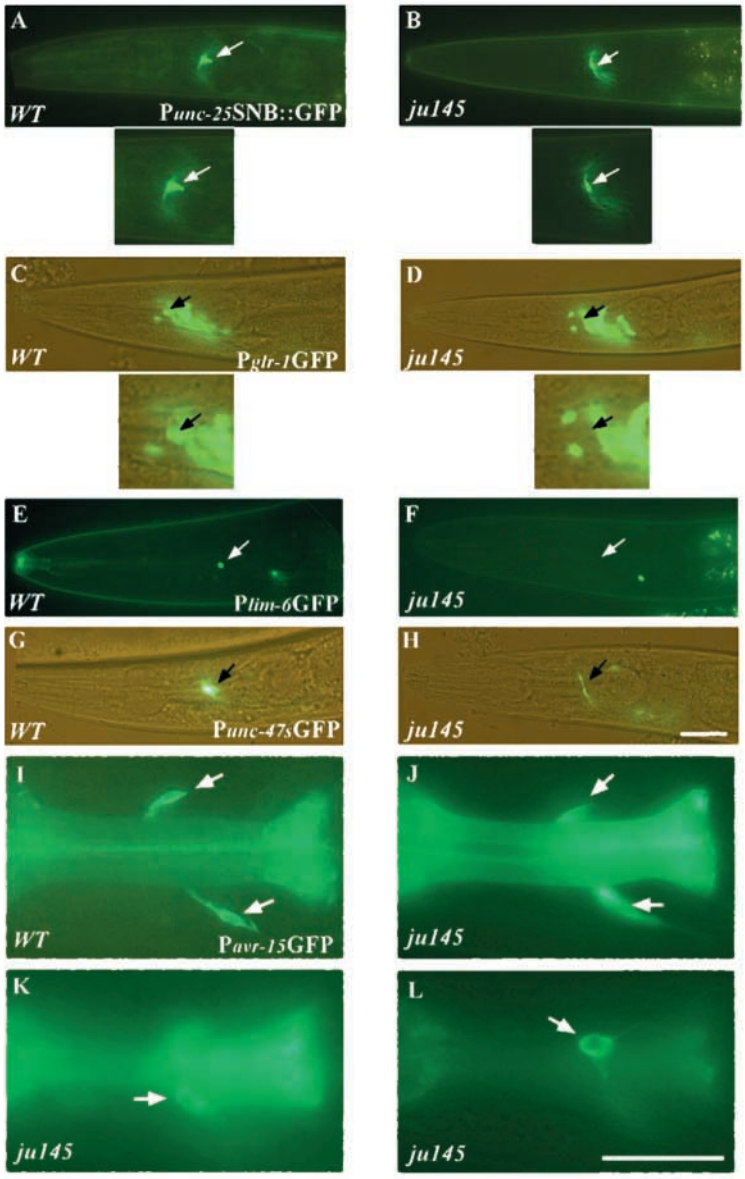

Fig. 3. *ju145* affects RMEL/R cell fate. (A,B) RMEL/R form fewer synapses (arrows) in *ju145* animals compared with wild type; enlarged views are shown below with arrows. (C-H) *ju145* mutation affects cell-specific marker expressions in RMEL/R neurons. Arrows point to the position of RMEL neurons. (C,D) P_{glr-1}GFP is expressed in RMEL/R neurons in wild type (C) but not in *ju145* (D) animals. (E,F) P_{lim-6r}GFP is expressed in RMEL/R neurons in wild type (E) but not *ju145* (F) animals. (G,H) $P_{unc-47s}$GFP is expressed in RMEL/R neurons in wild type (G) but not *ju145* animals. (I) P_{avr-15}GFP expression in RMED/V cells (arrows) in wild-type animals. (J-L) P_{avr-15}GFP is ectopically expressed in RMEL/R cells (arrows in K and L) in *ju145* animals; arrows in J indicate RMED/V cells. Scale bar: 20 μm.

The expression of *ahr-1* in RMEL/R and the mutant phenotypes of *ahr-1* suggest that *ahr-1* may act as a cell-type-specific determinant to control RMEL/R fate. To further test this hypothesis, we investigated the effect of ectopic *ahr-1* expression in RMED/V neurons. We used the *unc-25* promoter to express *ahr-1* in the four RMEs (see Materials and methods). The expression of *ahr-1* in RMEL/R from this transgene fully rescued the cell morphology defects of *ahr-1(ju145)* (Table 1), consistent with the conclusion that *ahr-1* functions in RMEL/R cells. Moreover, the ectopic expression of *ahr-1* in RMED/V repressed the formation and elongation of the longitudinal processes along the nerve cords in both wild-type animals and in *ahr-1(ju145)* animals (Fig. 5B, Table 1). To further address whether the ectopic expression of *ahr-1* causes RMED/V to adopt other differentiated features of RMEL/R cells, we analyzed the expression of P_{lim-6r}GFP in *juEx467[*P_{unc-25}*AHR-1]* transgenic animals. We found that in *juEx467* animals, in addition to its normal expression in RMEL/R, RMED/V cells also expressed P_{lim-6}GFP (Fig. 5C-F, Table 2). Thus, the ectopic expression of *ahr-1* in RMED/V cells can transform them into RMEL/R-like cells. These results strongly support the interpretation that *ahr-1* normally functions in RMEL/R cells to regulate their cell-specific gene expression.

aha-1/ARNT is required for *ahr-1* function in determining RMEL/R cell identity

It has been well established that *ahr-1* requires a nuclear cofactor ARNT (AHR nuclear translocator) to bind DNA (Reyes et al., 1992). The *C. elegans* ARNT AHA-1 is widely expressed (Powell-Coffman et al., 1998). A deletion mutation of *aha-1(ia1)* results in larval lethality that is due to its requirement in the pharynx. The larval lethality of *aha-1(ia1)* can be rescued by specific pharyngeal expression of *aha-1* (H. Jiang, S. Wu and J.A.P.-C., unpublished).

To address whether AHA-1 is required for AHR-1 function in RMEL/R cell fate specification, we introduced P_{unc-25}GFP into *aha-1(ia1)* mutants that are viable because of the pharyngeal expression of *aha-1*. We found that *aha-1(ia1)* animals showed identical RMEL/R cell morphology phenotypes to *ahr-1(ju145)* (Fig. 6C, Table 1), indicating that *aha-1* is required for RMEL/R cell fate. Furthermore, when we introduced into *aha-1(ia1)* mutants the *juEx467* transgene that expresses *ahr-1* ectopically in RMED/V cells, the RMED/V cells did not show inhibition of the longitudinal process extension (Fig. 6G,H; Table 1), supporting the conclusion that *aha-1* is required for the effect of ectopic *ahr-1*.

daf-21/Hsp90 is not required for *ahr-1* function in RMEL/R fate specification

Studies in yeast and mammalian cells have shown that the heat shock protein HSP90 is an essential chaperone to sequester AHRs in the cytoplasm (Pongratz et al., 1992). *daf-21* encodes *C. elegans* HSP90 and has been implicated in larval viability and dauer formation (Birnby et al., 2000). A null allele of *daf-21(nr2081)* results in larval lethality, and an unusual allele *daf-21(p673)* is viable to adulthood but causes constitutive dauer formation. We found that in both *daf-21(nr2081)* and *daf-21(p673)* animals, RMEL/R cell morphology was normal (Fig. 6D, Table 1), indicating that *daf-21* is unlikely to be required in RMEL/R neuron fate specification. Supporting this conclusion, *daf-21(p673)* showed no effects on the ectopic *ahr-*

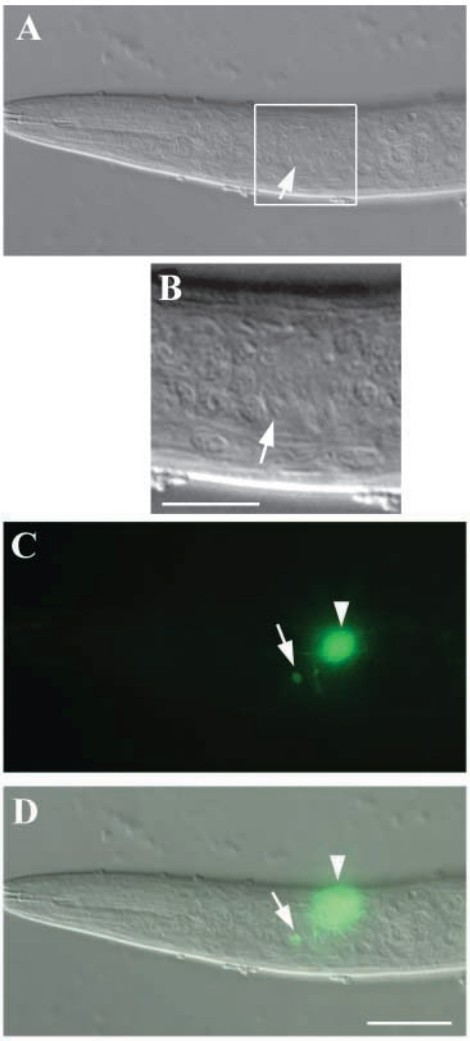

Fig. 4. AHR-1 is expressed in RMEL/R cells. (A) Nomarski picture of the head of an animal, with an enlarged view shown in B. (C) Fluorescence image of $P_{ahr\text{-}1}$GFP expression. Overlay is shown D. Arrows indicate the RMEL neurons. $P_{ahr\text{-}1}$GFP is also brightly expressed in a pair of sensory neurons (arrowhead), likely to be ASKL/R. Scale bar: in A,C,D, 50 μm; in B, 20 μm.

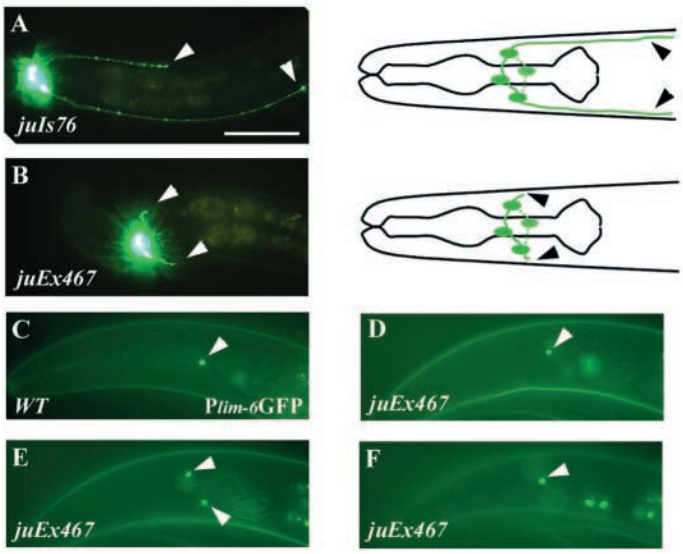

Fig. 5. Ectopic AHR-1 expression in RMED/V cells can transform them into RMEL/R-like cells. (A) *juIs76* wild-type animals. (B) Ectopic AHR-1 expression (*juEx467*) in RMED/V cells inhibits the growth of dorsal and ventral processes. Schematic illustrations are shown on the right. Arrowheads in A and B indicate the processes from REMD/V neurons. (C-F) Ectopic AHR-1 expression in RMED/V cells promotes $P_{lim\text{-}6}$GFP expression. (C) $P_{lim\text{-}6r}$GFP expression in a wild-type animal. Arrowhead indicates the RMEL neuron. (D-F) *juEx467* animals. Three focal planes are shown for $P_{lim\text{-}6r}$GFP expression in all four RME cells (arrowheads). Scale bar: 50 μm.

1 expression in RMED/V neurons (Fig. 6F, Table 1). It has been observed that a functional AHR-1::GFP is normally localized in the nucleus (H. Qin and J.A.P.-C., unpublished). These results suggest that unlike mammalian AHRs, *C. elegans* AHR-1 can be folded properly and localized to the correct cellular compartments independent of HSP90 chaperone function.

ahr-1, *ceh-10* and *lim-4* function independently to control RME neuron fate

The four RME neurons are closely related neurons by function and neurotransmitter specificity. The data presented above show that *ahr-1* regulates the cellular differentiation, but not GABA expression, of RMEL and RMER neurons. *lim-6* is expressed in RMEL, RMER and three other GABA neurons, and has been shown to partially regulate the expression of *unc-25* in AVL, DVB and RIS, but has no effect on RMEL/R (Hobert et al., 1999). To address whether *ahr-1* might function together with *lim-6* to control GABA expression, we made *ahr-1(ju145); lim-6(nr2073)* double mutants. These double mutants expressed $P_{unc\text{-}25}$GFP and $P_{unc\text{-}47}$GFP normally (data not shown). This observation, together with that that *ahr-1* regulates *lim-6* expression (Figs 3, 5), suggests that *lim-6* acts downstream of *ahr-1*, and mediates other functions of *ahr-1* in RMEL/R neurons.

Two transcription factors have been reported to be differentially expressed in RMED and RMEV neurons. *ceh-10* is expressed in RMED, and in *ceh-10* mutants RMED does not express GABA (Forrester et al., 1998). *lim-4* is expressed in RMEL (Sagasti et al., 1999). In a *lim-4* mutant, RMEL expresses GABA and has normal morphology (Fig. 7B, and data not shown). As ectopic expression of *ahr-1* is able to cause RMED/V neurons to adopt an RMEL/R-like fate, we addressed whether *ceh-10* and *lim-4* might repress the expression of *ahr-1* in RMED/V neurons. A functional AHR-1::GFP is expressed in the nuclei of a selected group of neurons including RMEL/R (H. Qin and J.A.P.-C., unpublished). We found that the expression of an integrated transgene expressing AHR-1::GFP was unaffected in *ceh-10(ct78)* and *lim-4(ky403)* animals (Fig. 7A). We further investigated how *ahr-1* might interact with *ceh-10* or *lim-4* by examining the RME neurons in pair-wise double mutants (Fig. 7B). We found that double mutants of *ahr-1(ju145); ceh-10(ct78)* showed an additive phenotype such that RMEL/R displayed ectopic nerve processes and RMED frequently failed to express $P_{unc\text{-}25}$GFP to the same degree as *ceh-10(ct78)* alone. Double mutants of *ahr-1(ju145); lim-4(ky403)*

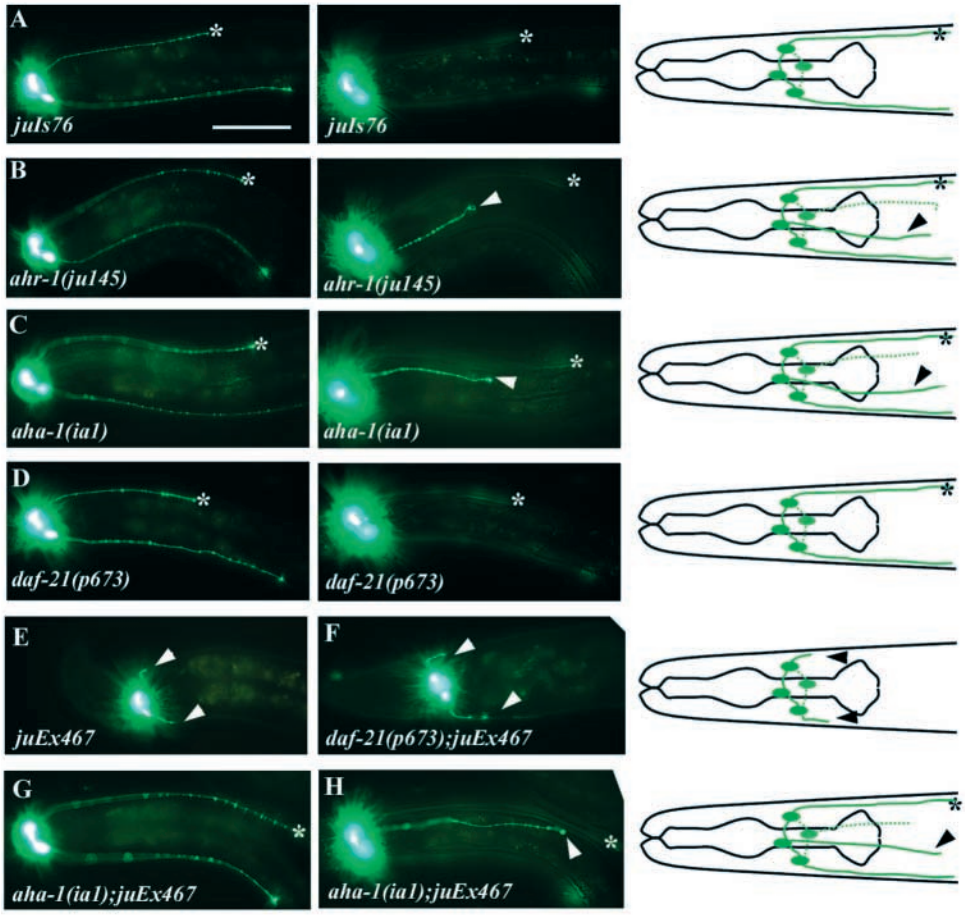

Fig. 6. *aha-1* but not *daf-21* is required for AHR-1 function in RMEL/R cells. (A-D) P_{unc-25}GFP expression in different mutants. Left and middle panels show two focal planes focused on RMED/V and RMEL neurons, respectively. Right panel shows the schematic illustration. Asterisk indicates process from RMED/V neurons; arrowheads, the ectopic processes from RMEL neurons. An *aha-1* mutant has the same RMEL phenotype (arrowheads) as an *ahr-1* mutant (B,C). In a *daf-21* mutant, RMEL and RMER neurons are normal (D). (E-H) Mutations in *aha-1* but not in *daf-21* suppress the effect of ectopic *ahr-1* expression in *juEx467*. Two focal planes are shown in G and H. Arrowheads in E and F indicate the processes from RMED/V neurons, and in H indicate the ectopic process from RMEL in *aha-1* mutants. Asterisk in G indicates the processes from RMED/V neurons. Scale bar: 50 μm.

exhibited only RMEL/R defects as did *ahr-1(ju145)* alone. Furthermore, in *ceh-10(ct78); lim-4(ky403)* double mutants, RMEV was normal, and RMED frequently failed to express P_{unc-25}GFP as did *ceh-10* alone. Thus, these observations are consistent with a conclusion that *ahr-1*, *ceh-10* and *lim-4* function independently of each other to control the development or function of the four RME neurons.

Discussion

C. elegans AHR-1 is a cell-type-specific determinant to control the fate of RMEL and RMER neurons

AHRs and the partner ARNTs are evolutionarily conserved sequence-specific DNA binding proteins, and are thus far extensively studied for their functions in toxin response in mammals (Hahn, 2002). *C. elegans ahr-1* is expressed in selected groups of neurons (H. Qin and J.A.P.-C., unpublished), two of which we identify as RMEL and RMER neurons. We show that in *ahr-1* loss-of-function mutants, RMEL/R cells extend ectopic axons and fail to express RMEL/R specific markers, although the expression and vesicular uptake of GABA in these neurons are normal. The altered RMEL/R neurons assume a morphology that resembles that of RMED/V neurons, and a RMED/V specific marker P_{avr-15}GFP is ectopically expressed in RMEL/R cells in *ahr-1* mutants. These results led us to conclude that loss of *ahr-1* function causes RMEL and RMER neurons to adopt a RMED/V-like fate. Furthermore, we show that the ectopic expression of *ahr-1* in RMED/V cells represses neurite extension and induces the expression of a RMEL/R-specific marker, P_{lim-6r}GFP. These observations indicate that *ahr-1* acts as a cell-type-specific factor to control RMEL/R fate specification.

This function of AHR-1 requires its co-factor AHA-1. *aha-1* is ubiquitously expressed (Jiang et al., 2001). We find that loss of function in *aha-1* causes morphological defects in RMEL/R neurons identical to those in *ahr-1* mutants, and that the ectopic effect of *ahr-1* requires *aha-1*. An independent study has found that several *ahr-1* expressing neurons exhibit aberrant cell morphology and cell migration defects in *ahr-1* mutants (H. Qin and J.A.P.-C., unpublished data). Thus, it is the cell-type-specific expression of *ahr-1* that allows the AHR-1/AHA-1 heterodimer to exert specific effects on neuronal differentiation.

GABAergic neuron fate specification in *C. elegans* involves multiple cellular determinants and signaling pathways

The twenty-six GABAergic neurons in *C. elegans* fall into five classes based on morphology and function (McIntire et al., 1993). The data presented here, together with previous published reports (Jin et al., 1994; Forrester et al., 1998; Hobert et al., 1999), classify them into additional subtypes and reveals different themes of regulatory network underlying the specification of each subtype. The ventral cord type D motor neurons are related in cell lineage, morphology and function. They express the UNC-30 homeodomain protein (Jin et al., 1994). UNC-30 co-regulates the expression of *unc-25* and *unc-47*, along with other unknown targets, to control multiple differentiation events in the type D neurons (Eastman et al., 1999).

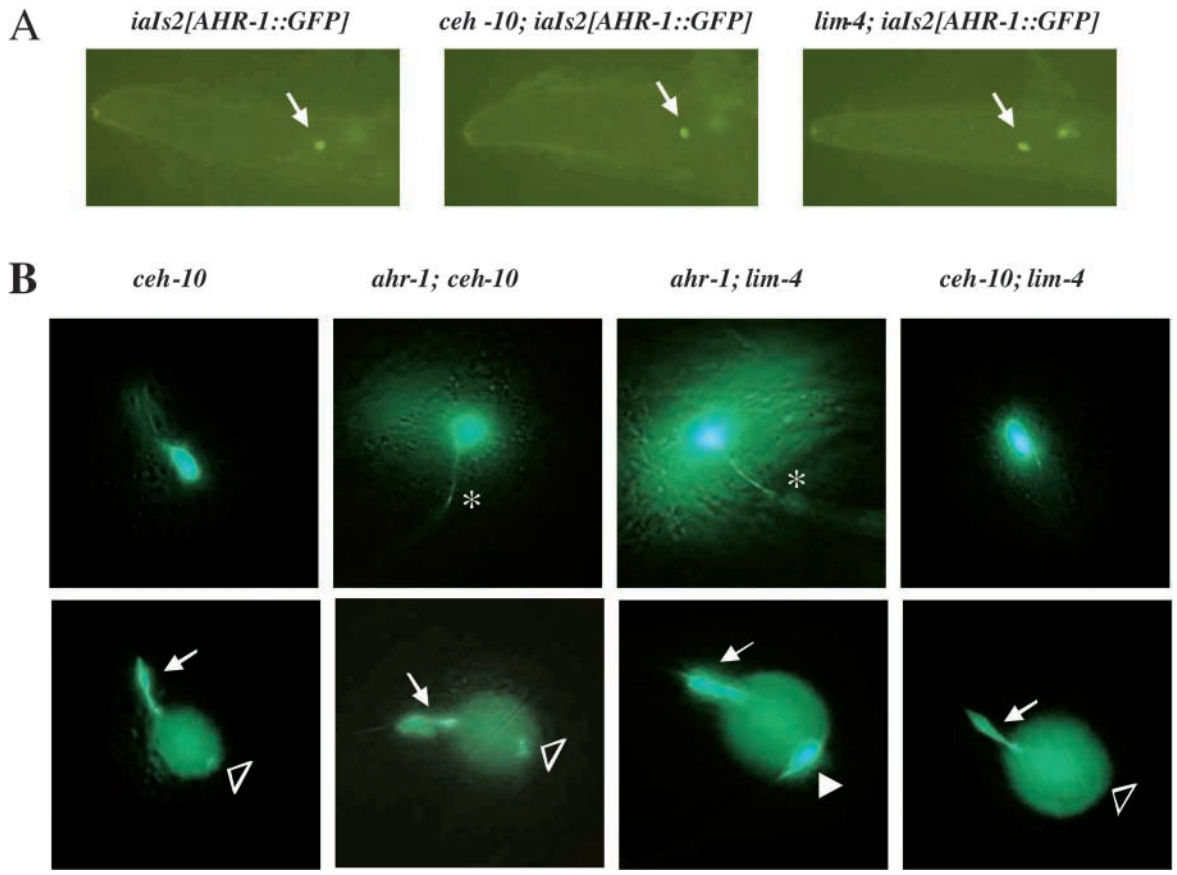

Fig. 7. *ahr-1*, *ceh-10* and *lim-4* function independently of each other in RME neurons. (A) Expression of a functional AHR-1::GFP (*iaIs2*) is unaltered in *ceh-10* and *lim-4* mutants. Arrows indicate RMEL in the three panels. Not all AHR-1 expressing neurons are in focus. (B) Double mutants of *ahr-1*, *ceh-10* or *lim-4* show additive phenotypes. Genotypes are shown above the panels. The top panels show RMEL/R neurons and the bottom panels show RMED/V neurons. Asterisks indicate the ectopic nerve process of RMEL/R; arrows indicate RMEV; solid arrowhead indicates RMED in *ahr-1, lim-4* double mutants; empty triangles show the presumed RMED position; and the fluorescence spots are from the distal ends of RMEV. More than 100 animals were observed for each genotype.

By contrast, the specification of the other seven GABA neurons depends on both cell-specific and common factors. The LIM-6 transcription factor is expressed in five GABA neurons, but has differential effects on their differentiation. In *lim-6* mutants, RMEL and RMER exhibit no discernible abnormality, but AVL and DVB display variable defects in neurite outgrowth and GABA expression, but not GABA vesicular packaging (Hobert et al., 1999). AHR-1 acts as a cell-type-specific determinant to control the cellular morphogenesis, but not neurotransmitter expression and packaging, of RMEL and RMER. The function of AHR-1 depends on its ubiquitously expressed cofactor AHA-1. *lim-6* is probably a downstream target of *ahr-1*, but *lim-6* is unlikely to mediate all functions of *ahr-1* in RMEL/R neurons because neither *glr-1* nor GABA expression is altered in *lim-6* mutants (Hobert et al., 1999) (this study).

Our observation that ectopic *ahr-1* expression can transform RMED/V to a RMEL/R-like fate further suggests that RMED/V cells may express some cellular determinant(s) that represses *ahr-1* expression and promotes their fate specification. Although *ceh-10* and *lim-4* are expressed in RMED and RMEV, respectively (Forrester et al., 1998; Hobert et al., 1999), we find that they do not function by repressing *ahr-1* expression in RMED/V. The analysis of double mutants among *ahr-1*, *ceh-10* and *lim-4* is also consistent with the conclusion that the specification of the four RME neurons involves parallel signaling pathways that may use multiple transcription cascades.

An ancestral role of AHR proteins is in regulating cell identity

All vertebrate AHRs bind dioxins through their PAS domains, and normally reside in the cytoplasm in a complex with HSP90 and XAP2 chaperonins (Petrulis and Perdew, 2002). Dioxin binding induces nuclear translocation, allowing the formation of AHR/ARNT heterodimer (Hahn, 2002). Supporting the role of AHRs in toxin response, AHR-deficient mice are resistant to many of the deleterious effects of AHR-activating pollutants (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Shimizu et al., 2000; Matikainen et al., 2001). However, these mice also show defects in liver, heart, ovary, vascular and immune systems, and have reduced growth, reproduction and survival, suggesting that AHR has important developmental functions, of which little is understood. In zebrafish embryos, exposure to TCDD increases apoptosis in the dorsal midbrain (Dong et al., 2001). In rat, AHR is expressed in the preoptic

area of the brain and co-localizes with GAD67, suggesting that the GABAergic neurons may be the cellular targets of TCDD-induced AHR function (Hays et al., 2002).

The *C. elegans* AHR-1 and *Drosophila* AHR Spineless (SS) have a PAS domain with divergent sequences and do not bind dioxin (Powell-Coffman et al., 1998; Butler et al., 2001). In *Drosophila ss* mutants, the distal antenna is transformed to distal leg, and most of the tarsal region of each leg is deleted (Duncan et al., 1998). SS is expressed in the tissues that are affected. Ectopic expression of *ss* can induce ectopic antennal structures from some tissues, indicating that *ss* functions as a tissue-specific factor to control distal antennal identity. Thus, studies in *Drosophila* and those reported here reveal an evolutionarily conserved function of AHRs as cell-type-specific determinants in animal development.

How might AHR signaling have evolved? At least two AHR-1 partners have been identified. One is ARNT, a ubiquitously expressed nuclear bHLH-PAS protein, and the other is the cytoplasmic chaperonin HSP90 (Hahn, 2002). The ARNT knockout mice died early, precluding the analysis of its involvement in AHR developmental function (Kozak et al., 1997). The *Drosophila* ARNT *Tango* is required for controlling antennal identity like *ss* (Emmons et al., 1999). We show that *C. elegans* AHA-1 is required for AHR-1 in controlling RMEL/R cell identity. Therefore, the AHR and ARNT are evolutionarily conserved functional partners in regulating developmental processes.

Studies in yeast and mammalian cells have implicated two roles of HSP90 in AHR function (Pongratz et al., 1992). HSP90 sequesters AHRs in the cytoplasm in the absence of ligands and also aids the proper folding of AHRs for ligand binding. The in vivo functional involvement of HSP90 in AHR toxin response has not been established. Although *C. elegans* AHR-1 can bind rabbit HSP90 in vitro (Powell-Coffman et al., 1998), AHR-1 is normally localized to nucleus (H. Qin and J.A.P.-C., unpublished). We find that *daf-21/Hsp90* is not required for RMEL/R cell identity, nor is it required for ectopic AHR-1 function, implying that AHR-1 can fold properly in the absence of HSP90.

Drosophila SS and Tango are both normally localized to the nucleus, and the nuclear localization of Tango depends on SS and other bHLH-PAS factors (Ward et al., 1998; Emmons et al., 1999). Similarly, the nuclear localization of *C. elegans* AHA-1 in intestinal cells is dependent on the co-expression of a bHLH-PAS dimerization partner HIF-1 (Jiang et al., 2001). The studies in invertebrates thus support a conclusion that the subcellular localization of AHRs and ARNT is achieved through co-expression of protein binding partners, not by ligand-induced nuclear translocation (Crews and Fan, 1999). Interestingly, expression studies in yeast show that the domains of *C. elegans* AHR-1 predicted to bind HSP90 and ligand exert a repressive function to inhibit nuclear translocation or transcriptional activation of AHR-1 (Powell-Coffman et al., 1998). Thus, one may speculate that the toxin response of AHRs in vertebrate animals evolved through a recently acquired interaction with HSP90 or other chaperonins, which confers the toxin-inducible feature of the mammalian AHRs.

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