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-Salgueiro et al., 1995; Schmidt et al., 1996) (for a review, see Gonzalez and Fernandez-Salgueiro, 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-18 (p673) causes constitutive formation of dauer larvae.

The C. elegans nervous system is composed of 320 neurons (White et al., 1986), 26 of which use γ-aminobutyric 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 lin-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 irinexin 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, based on cell lineage and gene expression. For example, RMEL and RMER are lineally related, and express lin-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 irinexin 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.

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 Apal-SpeI genomic fragment into pSL1190. To make the pHT101 Pahr-JGF plasmid, a 5377 bp HindIII-BamHI 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 Punc-25AHR-1, the ahr-1 promoter was replaced by the unc-25 promoter at the BanI 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(sa1006) injected at 20 ng/µl. Over 10 transgenic lines expressing Punc-25AHR-1 were obtained, and all showed similar effects. juIs467(Punc-25AHR-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 3P-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-parafomaldehyde fixation procedure described by McIntire et al. (McIntire et al., 1992). GFP reporter expression was directly observed under a 63× objective of a Zeiss Axiostar 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 (RME) 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.
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 \textit{unc-25} promoter to visualize the RME neurons [the genotype of the marker is \textit{juIs76} \textit{unc-25::GFP}] (Huang et al., 2002) (Fig. 1B). \textit{unc-25} encodes glutamic acid decarboxylase/GAD (Jin et al., 1999). In a visual screen for abnormal expression patterns of \textit{juIs76} \textit{unc-25::GFP}, we isolated the \textit{ju145} mutation because the mutant animals showed specific defects in the RMEL and RMER, but not RMED and RMEV (Table 1; and see below).

We mapped \textit{ju145} to chromosome I between \textit{unc-29} and \textit{dpy-24}, close to a polymorphism \textit{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: \textit{ahr-1} (C41G7.5), the \textit{C. elegans} ortholog of the aryl hydrocarbon receptor (Powell-Coffman et al., 1998), and C41G7.6, which resides in the third intron of \textit{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 \textit{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 \textit{ju145} animals by expressing \textit{ahr-1} specifically in RMEL/R neurons.

\textbf{RMEL and RMER neurons adopt an RMED/V-like fate in \textit{ahr-1} mutants}

In \textit{ahr-1(ju145)} animals the expression level of \textit{Punc-25::GFP} was normal in all four RME neurons. RMED and RMEV had normal cell morphology (Fig. 1B). However, RMEL and RMER exhibited aberrant 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). In \textit{ahr-1(ju145)} animals by expressing \textit{ahr-1} specifically in RMEL/R neurons. The RMEL/R defects remained the same in \textit{ju145/Df} animals (see Materials and methods). These results are consistent with the conclusion that the RMEL/R neuron defects of \textit{ju145} animals are due to the complete loss of function of \textit{ahr-1}.

\begin{table}[h!]
\centering
\caption{RME neuron defects in \textit{ahr-1} and other mutants}
\begin{tabular}{lcccccc}
\hline
 & RMED & RMEV & RMEL & RMER & \textit{n} \\
\hline
Wild type & 0 & 0 & 0 & 0 & \textgreater 100 \\
\textit{ahr-1(ju145)} & 0 & 0 & 0 & 0 & \textgreater 100 \\
\textit{aha-1(ia1)} & 0 & 0 & 100 & 100 & 30 \\
\textit{def-21(p673)*} & 0 & 0 & 100 & 100 & 28 \\
\textit{Punc-25::AHR-1(juEx467)} & 100 & 100 & 0 & 0 & \textgreater 100 \\
\textit{ahr-1(ju145); juEx467} & 100 & 100 & 0 & 0 & 30 \\
\textit{aha-1(ia1); juEx467} & 0 & 0 & 100 & 100 & 35 \\
\textit{def-21(p673); juEx467} & 100 & 100 & 0 & 0 & 40 \\
\hline
\end{tabular}
\hrule
\end{table}

*daf-21(n2081) has a similar result.
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-25~GFP and *P* ~unc-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-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-1~GFP, whereas *P* ~glr-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-6~GFP, in RMEL/R was abolished in *ju145* animals (Fig. 3E,F). It has been reported that in *lim-6* mutants *P* ~glr-1~GFP expression is normal and RMEL/R neurons are normal (Hobert et al., 1999), indicating that the effect on *P* ~glr-1~GFP in *ahr-1(ju145)* animals is independent of *lim-6*. In addition, we found that the expression of a transgene *P* ~unc-25~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-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-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-15~GFP marker was now expressed in RMEL/R cells in *ju145* animals (Fig. 3J; 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

<table>
<thead>
<tr>
<th>Percentage of animals that express GFP</th>
<th>RMED</th>
<th>RMEV</th>
<th>RMEL</th>
<th>RMER</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P</em> <del>glr-1</del>GFP</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td><em>ahr-1</em>; <em>P</em> <del>glr-1</del>GFP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td><em>P</em> <del>unc-25</del>GFP</td>
<td>0</td>
<td>0</td>
<td>98</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td><em>ahr-1</em>; <em>P</em> <del>unc-25</del>GFP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td><em>juEx467</em>; <em>P</em> <del>unc-47</del>GFP</td>
<td>88</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td><em>P</em> <del>unc-47</del>GFP</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td><em>ahr-1</em>; <em>P</em> <del>unc-47</del>GFP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td><em>P</em> <del>avr-15</del>GFP</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td><em>ahr-1</em>; <em>P</em> <del>avr-15</del>GFP</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>100</td>
<td>38</td>
</tr>
</tbody>
</table>

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).
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*$_{lim-6}$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-1* expression.
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 Punc-25GFP and Punc-47GFP 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 Punc-25GFP to the same degree as ceh-10(ct78) alone. Double mutants of ahr-1(ju145); lim-4(ky403)
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 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. Nevertheless, 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).
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 \textit{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 RME. The function of AHR-1 depends on its ubiquitously expressed cofactor AHA-1. \textit{lim-6} is probably a downstream target of \textit{ahr-1}, but \textit{lim-6} is unlikely to mediate all functions of \textit{ahr-1} in RMEL/R neurons because neither \textit{glr-1} nor GABA expression is altered in \textit{lim-6} mutants (Hobert et al., 1999) (this study).

Our observation that ectopic \textit{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 \textit{ahr-1} expression and promotes their fate specification. Although \textit{ceh-10} and \textit{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 \textit{ahr-1} expression in RMED/V. The analysis of double mutants among \textit{ahr-1}, \textit{ceh-10} and \textit{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 (Petruis 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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