Neuronal functions of *ahr-1*, the *Caenorhabditis elegans* homolog of the aryl hydrocarbon receptor

by

Hongtao Qin

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Jo Anne Powell-Coffman, Major Professor
Jack R. Girton
Kristen M. Johansen
James M. Reecy
Donald S. Sakaguchi

Iowa State University

Ames, Iowa

2006

Graduate College

Iowa State University

This is to certify that the doctoral dissertation of

Hongtao Qin

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

TABLE OF CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

Dissertation Organization

This dissertation presents the study of aryl hydrocarbon receptor in a powerful genetic model system, the nematode *Caenorhabditis elegans*. This study demonstrates that *Caenorhabditis elegans* aryl hydrocarbon receptor gene, *ahr-1*, plays important roles in regulating neuronal development and neuronal function. This dissertation is composed of 4 chapters. In Chapter 1, I briefly introduce background information that is necessary for the readers to understand the rational and significance of this study. Chapter 2 is a journal article that was published in *Developmental Biology, 2004, 207(1):64-75*. The title is "The *Caenorhabditis elegans* aryl hydrocarbon receptor, AHR-1, regulates neuronal development". In this paper, I present evidence demonstrating that *ahr-1* is expressed exclusively in the neural system and regulates neuronal development. Jo Anne Powell-Coffman created the *ahr-1 (ia03)* deletion allele and pJ360 construct for this study. Chapter 3 is a research paper that has been submitted. The title is "The *Caenorhabditis elegans* aryl hydrocarbon receptor controls expression of soluble guanylate cyclases in the URX neurons and regulates social feeding behavior". This paper demonstrates that *ahr-1* controls the expression of 5 soluble guanylate cyclases in URX neurons and we provide evidence that *ahr-1* functions acutely in URX to promote aggregation behavior. Other researchers in the laboratory were also involved in this study. Zhiwei Zhai constructed *Pgcy-37:GFP* plasmid pZW2. Jo Anne Powell-Coffman contributed to experimental design and to writing reports for publication or funding. In Chapter 4, I summarize the major finding of my Ph.D. work, discuss the impact of this work to related research fields, and provide insights to the prospects of *ahr-1* studies.

Background

AHR as a xenobiotic sensor

The aryl hydrocarbon receptor (AHR) contains basic helix-loop-helix and PAS domains and has important biological functions. AHR is well-known as a ligand activated transcription factor and it mediates the adaptation responses and toxic responses for many important environment pollutants (Crews, 2003). AHR ligands include polycyclic aromatic hydrocarbons (PAH) and certain halogenated aromatic hydrocarbons (HAH). Activated by PAHs, AHR induces the expression of a battery of xenobiotic metabolizing enzymes (XMEs) that can metabolize PAHs, including cytochrome P450 CYP1A1, CYP1A2, and CYP1B1 as well as the phase II enzymes GST-A1 and UGT1-06 (Schmidt and Bradfield, 1996; Whitlock, 1999). This adaptation response to PAHs could be deleterious, because the intermediate metabolites can mutate DNA and therefore are carcinogenic (Stansbury et al., 1994). AHR null mice are resistant to the carcinogenicity of benzo[a]pyrene, supporting the conclusion that AHR mediates the carcinogenic activity of PAHs (Shimizu et al., 2000). Unlike PAHs, the most potent HAH 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is resistant to the metabolism of cytochrome P450s (Nebert et al., 2000). The half-life of TCDD in human tissues is approximately 7 years (Pirkle et al., 1989). Exposure to dioxin causes pleiotropic defects in a variety of vertebrates including birth defects, cancer, hepatotoxicity, immunological deficiencies, cognitive impairment, reduction of feed intake, and death (Pohjanvirta and Tuomisto, 1994; Birnbaum and Tuomisto, 2000; Mandal, 2005). The AHR knockout mice are resistant to many of the deleterious effects of TCDD and related compounds (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Shimizu et al., 2000; Matikainen et al., 2001; Vorderstrasse et al., 2001). Most of the toxic effects of TCDD are mediated by AHR (Hankinson, 1995; Schmidt and Bradfield, 1996; Mandal, 2005).

The molecular mechanisms by which AHR mediates toxic effects have been extensively studied. AHR and its nuclear dimerization partner, ARNT, are both members

of an evolutionarily conserved family of transcription factors that contain an N-terminal basic-helix-loop-helix (bHLH) DNA-binding and dimerization motif, and a PAS domain, named for its discovery in Drosophila Period, mammalian ARNT, and Drosophila Singleminded (Crews and Fan, 1999; Gu et al., 2000). bHLH-PAS proteins control critical cellular and developmental processes, including neuronal differentiation, circadian rhythm regulation and response to hypoxia. In the absence of ligand, AHR resides in the cytoplasm in a complex with 90 kDa heat shock proteins (HSP90), AHR-associated protein 9 (ARA9), p23 and additional chaperonins (Denis et al., 1988; Perdew, 1988; Carver and Bradfield, 1997; Ma and Whitlock, 1997; Carver et al., 1998; Meyer et al., 2000; Petrulis and Perdew, 2002). Upon binding ligand, the receptor translocates to the nucleus, dissociates from HSP90, and forms heterodimers with ARNT (Reyes et al., 1992; Probst et al., 1993). The AHR:ARNT complex binds to specific DNA sequences, termed xenobiotic response elements (XREs, 5' -G/TNGCGTGA/C- 3'), to regulate the expression of target genes (Hankinson, 1995; Swanson, 2002). It is not fully understood how the inappropriate expression of these target genes causes specific toxic effects of TCDD (Dalton et al., 2002; Mimura and Fujii-Kuriyama, 2003; Uno et al., 2004; Xu et al., 2005).

AHR endogenous function

Although the studies of the AHR have been strongly biased to understanding its xenobiotic sensor functions, several lines of evidence have demonstrated that AHR has important endogenous functions. First, the toxicities of TCDD and related compounds suggest that these chemicals are interfering with important physiological and developmental functions in addition to inducing xenobiotic metabolizing enzymes. AHR mediates most of these toxic effects of TCDD and related compounds (Hankinson, 1995; Schmidt and Bradfield, 1996; Mandal, 2005). This suggests that AHR might have important endogenous functions that regulate important developmental events and physiology.

Second, numerous studies have shown that AHR signaling can be activated in the absence of exogenous ligands, as evidenced by the presence of nuclear AHR complex and

AHR-dependent response in ligand-untreated animals and cell cultures (Chang and Puga, 1998; Denison et al., 2002; Puga et al., 2002; Oesch-Bartlomowicz et al., 2005). The AHR signaling could be activated by AHR endogenous ligands, or AHR may have ligand-independent endogenous functions. Several endogenous chemicals have been identified that can bind to AHR and/or activate AHR-dependent gene expression (Denison and Nagy, 2003). However, it has not yet been demonstrated that these endogenous ligand candidates regulate AHR function *in vivo*.

Third, AHR homologs have been identified in a variety of metazoan species, including vertebrates and invertebrates (Hahn, 2002). AHR orthologs exist in many living vertebrate representatives including mammals, reptiles, birds, amphibians and fish (Hahn, 2001). Although living in very different chemical environments, many vertebrate species are sensitive to TCDD and/or PAHs . The AHR of these species binds to TCDD and mediates the cellular responses this toxic compound (Hahn, 2002). The conservation of AHR in these vertebrate species suggests other important endogenous roles of AHR in addition to sensing TCDD and other environmental chemicals. Invertebrate AHR has been found in *C. elegans, D. melanogaster*, and molluscs (Duncan et al., 1998; Powell-Coffman et al., 1998; Butler et al., 2001; Moore, 2002). These invertebrate AHR homologs contain highly conserved bHLH and PAS domains, form protein complex with invertebrate ARNT, and bind to conserved XRE sequences (Powell-Coffman et al., 1998; Emmons et al., 1999; Butler et al., 2001). Interestingly, none of the invertebrate AHR protein specifically binds to prototypical AHR ligands [^{3}H]TCDD and [^{3}H]BNF (Powell-Coffman et al., 1998; Butler et al., 2001). Instead, the function of either *C. elegans* AHR or *Drosophila* AHR is required to regulate normal development (Duncan et al., 1998; Huang et al., 2004; Qin and Powell-Coffman, 2004). Phylogenetic studies suggest that the original function of ancestral AHR was to regulate animal development, and the xenobiotic sensor functions might have first evolved in early jawed vertebrates (Hahn, 2002).

Fourth, four murine AHR alleles have been identified, including Ah^d, Ah^{b-1}, Ah^{b-2} and Ah^{b-3} (Poland and Glover, 1980; Ema et al., 1994; Poland et al., 1994). Compared with other alleles, Ah^d has lower binding affinity to a photoaffinity ligand (^{125}I)-2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin, and Ah^d mice are less sensitive to PAHs (Poland and Glover, 1990; Poland et al., 1994). Sequence analyses identified an amino acid polymorphism in the ligand binding domain. A point mutation $Ala^{375} \rightarrow Val^{375}$ in Ah^d correlates with its low ligand binding affinity (Chang et al., 1993; Ema et al., 1994; Poland et al., 1994). However, no obvious physiological defects were noticed in the Ah^d mice (Crews, 2003). This suggests that AHR xenobiotic sensor function is not essential and AHR might have an endogenous function.

Finally, the strongest evidence that AHR has important endogenous roles derives from the analyses of AHR knockout mice. Several labs have independently developed AHR null mice strains by using gene targeting approaches (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997). As expected, these AHR-deficient mice are not responsive to AHR agonists and are not sensitive to TCDD (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997; Peters et al., 1999; Shimizu et al., 2000; Vorderstrasse et al., 2001). Although the reported phenotypes of AHR null mice are varied, all of the null strains exhibit defects in liver development, decreased body size, and poor fertility (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997; Lahvis and Bradfield, 1998; Benedict et al., 2000; Lahvis et al., 2000). Defects in hepatic vascular development were reported in AHR and ARNT hypomorphs (Walisser et al., 2004). Gestational exposure of these hypomorphs to dioxin between embryonic day 12.5 and embryonic day 18.5 can rescue the defects in hepatic vascular development (Walisser et al., 2004). This suggests that AHR signaling during embryonic development is required to regulate vascular development. Recently, Walisser *et al.* (2005) shows that AHR's liver development function and hepatotoxicity function can be uncoupled into different cell types. AHR functions in endothelial hematopoietic cells to regulate the vascular development,

whereas AHR is required in hepatocytes to generate toxic responses of the liver to dioxin exposure (Walisser et al., 2005).

AHR functions in the neural system to mediate dioxin toxicities

AHR is expressed in many tissues, including the brain (Abbott et al., 1995; Jain et al., 1998; Petersen et al., 2000). Dioxin, the most potent known AHR agonist, and related compounds can dramatically impair brain physiology and development. One of the characteristics of acute TCDD toxicity is progressive hypophagia that results in body weight loss (Pohjanvirta and Tuomisto, 1994). The early anorexia and decline of weight of TCDD treated rats are caused by depressed motivation to eat (Pohjanvirta and Tuomisto, 1994; Unkila et al., 1995; Tuomisto et al., 1999). Lethal doses of TCDD affect the metabolism of serotonin, a neurotransmitter that is capable to regulate food intake (Unkila et al., 1995). This suggests that the central nervous system is a target of TCDD toxicity. The mechanisms by which these TCDD toxicities are mediated are not understood. Toxicological studies also suggest that inappropriate activation of AHR can alter cell fates in the developing nervous system. Fetal exposure to TCDD can cause a range of neurological defects in humans and other primates, including reduced cognitive abilities and altered reproductive behaviors (Schantz and Bowman, 1989; Schantz et al., 1992; Peterson et al., 1993; Patandin et al., 1999). AHR-activating ligands have been shown to cause abnormal apoptosis in the zebrafish brain and asymmetrical brain development in the chick (Henshel et al., 1997; Dong et al., 2001). TCDD exposure also minimizes sex-specific differences in gene expression in certain rat GABAergic neurons (Hays et al., 2002). However, the endogenous function of AHR in neural system is not understood.

C. elegans AHR

The *C. elegans* orthologs of AHR and ARNT are encoded by the *ahr-1* and *aha-1* genes (Powell-Coffman et al., 1998). Although they were first identified by sequence similarity, AHR-1 and AHA-1 share similar biochemical features with their mammalian cognates. AHR-1 and AHA-1 interact to bind DNA fragments containing xenobiotic

response elements (XRE, KNGCGTG) with sequence specificity, and AHR-1 interacts with the 90 kDa heat shock protein (Hsp90) *in vitro* (Powell-Coffman et al., 1998). AHR-1 also has specialized biochemical characteristics that are different from its mammalian cognate. AHR-1 does not interact with another mammalian AHR-associated chaperonin ARA9/AIP1/XAP2 (Bell and Poland, 2000). Like other invertebrate AHR homologs that have been tested, AHR-1 does not bind to AHR ligand TCDD or β-naphthoflavone (Powell-Coffman et al., 1998; Butler et al., 2001). No ligand of AHR-1 has been identified. This suggests that AHR-1 does not possess a xenobiotic sensor function as its mammalian cognate, and may have important endogenous functions. AHR-1 endogenous functions may be ligand-independent or may be regulated by unknown ligands. Yeast expression assays suggest that AHR-1 is not constitutively activated and that nuclear localization or transcriptional activation of AHR-1 requires post-transcription modification (Powell-Coffman et al., 1998). Understanding AHR-1 function and regulation in *C. elegans* model system will provide great insight into the studies of AHR endogenous function.

C. elegans as a model system for studying neural development and behavior

The nematode *C. elegans* is a powerful genetic model organism for studying neural development and behavior. *C. elegans* strains can be cultured conveniently on agar plates with bacterial food and maintained as frozen stocks (Wood, 1988). Because of the small size (1 mm as an adult hermaphrodite), short generation time (3 days at 20°C), and the existence of both males and self-fertilizing hermaphrodites, *C. elegans* has considerable advantages for genetic analyses (Wood, 1988). High density single-nucleotide polymorphism (SNP) maps of different *C. elegans* isolates facilitate positional cloning (Jakubowski and Kornfeld, 1999; Koch et al., 2000). The *C. elegans* genome has been completely sequenced and well annotated (http://www.wormbase.org/). Genome-scale studies are underway to characterize the expression and function of every gene by RNA interference, microarrays, RNA *in situ* hybridization and yeast two-hybrid, and the data are

deposited in publicly accessible databases (Tabara et al., 1996; Kim et al., 2001; Boulton et al., 2002; Grant and Wilkinson, 2003; Kamath et al., 2003; Vaglio et al., 2003).

The nervous system of an adult *C. elegans* hermaphrodite consists of 302 neurons. The morphology, chemical synapses, and gap junctions of all 302 neurons have been determined by reconstruction of electron micrographs of serial sections (Albertson and Thomson, 1976; White et al., 1976, , 1986; Durbin, 1987). Based on morphology and connectivity, 302 neurons are classified into at least 118 neuronal subtypes, with 1 to 13 members in each group (White et al., 1986). The entire cell lineage of *C. elegans* has been determined (Sulston and Horvitz, 1977; Sulston et al., 1983). The high resolution maps of neural anatomy and powerful genetic and molecular methods make it relatively easy to study cell type specific gene function and neuronal development. Although the neuronal system of *C. elegans* is relative simple, the worms exhibit a rich behavioral repertoire (Hobert, 2003; de Bono and Maricq, 2005). Many reliable behavior assays have been developed. Genetic screens for *C. elegans* behavior mutants have identified genes that are required for the sensory signaling machines (receptors, channels, neurotransmitter), and the development of groups of neurons that underlie specific behaviors (Hobert, 2003). Genes that regulate behavioral plasticity without affecting neural development were also identified (Hobert, 2003).

Summary

In addition to its roles in the adaptive response to PAHs and the pleiotropic toxic response to dioxin, mammalian AHR possesses important endogenous functions to regulate normal development and presumably physiological events. In recent years, it has become clear that fully understanding the pleiotropic toxic effects of AHR ligands requires a better understanding of the endogenous function of AHR (Crews, 2003). Toxicological studies have shown that AHR functions in the neural system to mediate the toxic effects of TCDD. However, the endogenous functions of AHR in the neural system are not understood. *C.*

elegans provides an attractive experimental system to study the function and regulation of ancestral AHR in the neural system.

Reference

Abbott BD, Birnbaum LS, Perdew GH (1995) Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. Dev Dyn 204:133-143.

Albertson DG, Thomson JN (1976) The pharynx of Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci 275:299-325.

Bell DR, Poland A (2000) Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein. The role of hsp90. J Biol Chem 275:36407-36414.

Benedict JC, Lin TM, Loeffler IK, Peterson RE, Flaws JA (2000) Physiological role of the aryl hydrocarbon receptor in mouse ovary development. Toxicol Sci 56:382-388.

Birnbaum LS, Tuomisto J (2000) Non-carcinogenic effects of TCDD in animals. Food Addit Contam 17:275-288.

Boulton SJ, Gartner A, Reboul J, Vaglio P, Dyson N, Hill DE, Vidal M (2002) Combined functional genomic maps of the C. elegans DNA damage response. Science 295:127-131.

Butler RA, Kelley ML, Powell WH, Hahn ME, Van Beneden RJ (2001) An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, Mya arenaria: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone binding. Gene 278:223-234.

Carver LA, Bradfield CA (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. J Biol Chem 272:11452-11456.

Carver LA, LaPres JJ, Jain S, Dunham EE, Bradfield CA (1998) Characterization of the Ah receptor-associated protein, ARA9. J Biol Chem 273:33580-33587.

Chang C, Smith DR, Prasad VS, Sidman CL, Nebert DW, Puga A (1993) Ten nucleotide differences, five of which cause amino acid changes, are associated with the Ah receptor locus polymorphism of C57BL/6 and DBA/2 mice. Pharmacogenetics 3:312-321.

Chang CY, Puga A (1998) Constitutive activation of the aromatic hydrocarbon receptor. Mol Cell Biol 18:525-535.

Crews SE (2003) PAS proteins : regulators and sensors of development and physiology. Boston: Kluwer Academic Publishers.

Crews ST, Fan CM (1999) Remembrance of things PAS: regulation of development by bHLH-PAS proteins. Curr Opin Genet Dev 9:580-587.

Dalton TP, Puga A, Shertzer HG (2002) Induction of cellular oxidative stress by aryl hydrocarbon receptor activation. Chem Biol Interact 141:77-95.

de Bono M, Maricq AV (2005) Neuronal substrates of complex behaviors in C. elegans. Annu Rev Neurosci 28:451-501.

Denis M, Cuthill S, Wikstrom AC, Poellinger L, Gustafsson JA (1988) Association of the dioxin receptor with the Mr 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor. Biochem Biophys Res Commun 155:801-807.

Denison MS, Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43:309-334.

Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L (2002) Ligand binding and activation of the Ah receptor. Chem Biol Interact 141:3-24.

Dong W, Teraoka H, Kondo S, Hiraga T (2001) 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. Neurosci Lett 303:169-172.

Duncan DM, Burgess EA, Duncan I (1998) Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev 12:1290-1303.

Durbin R (1987) Studies on the development and organisation of the nervous system of *Caenorhabditis elegans*. In. Cambridge, England: University of Cambridge.

Ema M, Ohe N, Suzuki M, Mimura J, Sogawa K, Ikawa S, Fujii-Kuriyama Y (1994) Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. J Biol Chem 269:27337-27343.

Emmons RB, Duncan D, Estes PA, Kiefel P, Mosher JT, Sonnenfeld M, Ward MP, Duncan I, Crews ST (1999) The spineless-aristapedia and tango bHLH-PAS proteins interact to control antennal and tarsal development in Drosophila. Development 126:3937-3945.

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 268:722-726.

Grant BD, Wilkinson HA (2003) Functional genomic maps in Caenorhabditis elegans. Curr Opin Cell Biol 15:206-212.

Gu YZ, Hogenesch JB, Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol 40:519-561.

Hahn ME (2001) Dioxin toxicology and the aryl hydrocarbon receptor: insights from fish and other non-traditional models. Mar Biotechnol (NY) 3:S224-238.

Hahn ME (2002) Aryl hydrocarbon receptors: diversity and evolution. Chem Biol Interact 141:131-160.

Hankinson O (1995) The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35:307-340.

Hays LE, Carpenter CD, Petersen SL (2002) Evidence that GABAergic neurons in the preoptic area of the rat brain are targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin during development. Environ Health Perspect 110 Suppl 3:369-376.

Henshel DS, Martin JW, DeWitt JC (1997) Brain asymmetry as a potential biomarker for developmental TCDD intoxication: a dose-response study. Environ Health Perspect 105:718-725.

Hobert O (2003) Behavioral plasticity in C. elegans: paradigms, circuits, genes. J Neurobiol 54:203-223.

Huang X, Powell-Coffman JA, Jin Y (2004) 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. Development 131:819-828.

Jain S, Maltepe E, Lu MM, Simon C, Bradfield CA (1998) Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. Mech Dev 73:117-123.

Jakubowski J, Kornfeld K (1999) A local, high-density, single-nucleotide polymorphism map used to clone Caenorhabditis elegans cdf-1. Genetics 153:743-752.

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J (2003) Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 421:231-237.

Kim SK, Lund J, Kiraly M, Duke K, Jiang M, Stuart JM, Eizinger A, Wylie BN, Davidson GS (2001) A gene expression map for Caenorhabditis elegans. Science 293:2087-2092.

Koch R, van Luenen HG, van der Horst M, Thijssen KL, Plasterk RH (2000) Single nucleotide polymorphisms in wild isolates of Caenorhabditis elegans. Genome Res 10:1690-1696.

Lahvis GP, Bradfield CA (1998) Ahr null alleles: distinctive or different? Biochem Pharmacol 56:781-787.

Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, Bradfield CA (2000) Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. Proc Natl Acad Sci U S A 97:10442-10447.

Ma Q, Whitlock JP, Jr. (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem 272:8878-8884.

Mandal PK (2005) Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. J Comp Physiol [B] 175:221-230.

Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai T, Korsmeyer SJ, Casper RF, Sherr DH, Tilly JL (2001) Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. Nat Genet 28:355-360.

Meyer BK, Petrulis JR, Perdew GH (2000) Aryl hydrocarbon (Ah) receptor levels are selectively modulated by hsp90-associated immunophilin homolog XAP2. Cell Stress Chaperones 5:243-254.

Mimura J, Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta 1619:263-268.

Mimura J, Yamashita K, Nakamura K, Morita M, Takagi TN, Nakao K, Ema M, Sogawa K, Yasuda M, Katsuki M, Fujii-Kuriyama Y (1997) Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 2:645-654.

Moore MM (2002) Pollutant responses in marine organisms (PRIMO 11). Oxford: Elsevier.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP (2000) Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem Pharmacol 59:65-85.

Oesch-Bartlomowicz B, Huelster A, Wiss O, Antoniou-Lipfert P, Dietrich C, Arand M, Weiss C, Bockamp E, Oesch F (2005) Aryl hydrocarbon receptor activation by cAMP vs. dioxin: divergent signaling pathways. Proc Natl Acad Sci U S A 102:9218-9223.

Patandin S, Dagnelie PC, Mulder PG, Op de Coul E, van der Veen JE, Weisglas-Kuperus N, Sauer PJ (1999) Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: A comparison between breast-feeding, toddler, and long-term exposure. Environ Health Perspect 107:45-51.

Perdew GH (1988) Association of the Ah receptor with the 90-kDa heat shock protein. J Biol Chem 263:13802-13805.

Peters JM, Narotsky MG, Elizondo G, Fernandez-Salguero PM, Gonzalez FJ, Abbott BD (1999) Amelioration of TCDD-induced teratogenesis in aryl hydrocarbon receptor (AhR)-null mice. Toxicol Sci 47:86-92.

Petersen SL, Curran MA, Marconi SA, Carpenter CD, Lubbers LS, McAbee MD (2000) Distribution of mRNAs encoding the arylhydrocarbon receptor, arylhydrocarbon receptor nuclear translocator, and arylhydrocarbon receptor nuclear translocator-2 in the rat brain and brainstem. J Comp Neurol 427:428-439.

Peterson RE, Theobald HM, Kimmel GL (1993) Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol 23:283-335.

Petrulis JR, Perdew GH (2002) The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chem Biol Interact 141:25-40.

Pirkle JL, Wolfe WH, Patterson DG, Needham LL, Michalek JE, Miner JC, Peterson MR, Phillips DL (1989) Estimates of the half-life of 2,3,7,8-tetrachlorodibenzo-p-dioxin

in Vietnam Veterans of Operation Ranch Hand. J Toxicol Environ Health 27:165-171.

Pohjanvirta R, Tuomisto J (1994) Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: effects, mechanisms, and animal models. Pharmacol Rev 46:483-549.

Poland A, Glover E (1980) 2,3,7,8,-Tetrachlorodibenzo-p-dioxin: segregation of toxocity with the Ah locus. Mol Pharmacol 17:86-94.

Poland A, Glover E (1990) Characterization and strain distribution pattern of the murine Ah receptor specified by the Ahd and Ahb-3 alleles. Mol Pharmacol 38:306-312.

Poland A, Palen D, Glover E (1994) Analysis of the four alleles of the murine aryl hydrocarbon receptor. Mol Pharmacol 46:915-921.

Powell-Coffman JA, Bradfield CA, Wood WB (1998) Caenorhabditis elegans orthologs of the aryl hydrocarbon receptor and its heterodimerization partner the aryl hydrocarbon receptor nuclear translocator. Proc Natl Acad Sci U S A 95:2844-2849.

Probst MR, Reisz-Porszasz S, Agbunag RV, Ong MS, Hankinson O (1993) Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. Mol Pharmacol 44:511-518.

Puga A, Xia Y, Elferink C (2002) Role of the aryl hydrocarbon receptor in cell cycle regulation. Chem Biol Interact 141:117-130.

Qin H, Powell-Coffman JA (2004) The Caenorhabditis elegans aryl hydrocarbon receptor, AHR-1, regulates neuronal development. Dev Biol 270:64-75.

Reyes H, Reisz-Porszasz S, Hankinson O (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 256:1193-1195.

Schantz SL, Bowman RE (1989) Learning in monkeys exposed perinatally to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Neurotoxicol Teratol 11:13-19.

Schantz SL, Ferguson SA, Bowman RE (1992) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on behavior of monkeys in peer groups. Neurotoxicol Teratol 14:433-446.

Schmidt JV, Bradfield CA (1996) Ah receptor signaling pathways. Annu Rev Cell Dev Biol 12:55-89.

Schmidt JV, Su GH, Reddy JK, Simon MC, Bradfield CA (1996) Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. Proc Natl Acad Sci U S A 93:6731-6736.

Shimizu Y, Nakatsuru Y, Ichinose M, Takahashi Y, Kume H, Mimura J, Fujii-Kuriyama Y, Ishikawa T (2000) Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc Natl Acad Sci U S A 97:779-782.

Stansbury KH, Flesher JW, Gupta RC (1994) Mechanism of aralkyl-DNA adduct formation from benzo[a]pyrene in vivo. Chem Res Toxicol 7:254-259.

Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol 56:110-156.

Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol 100:64-119.

Swanson HI (2002) DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. Chem Biol Interact 141:63-76.

Tabara H, Motohashi T, Kohara Y (1996) A multi-well version of in situ hybridization on whole mount embryos of Caenorhabditis elegans. Nucleic Acids Res 24:2119-2124.

Tuomisto JT, Pohjanvirta R, Unkila M, Tuomisto J (1999) TCDD-induced anorexia and wasting syndrome in rats: effects of diet-induced obesity and nutrition. Pharmacol Biochem Behav 62:735-742.

Unkila M, Pohjanvirta R, Tuomisto J (1995) Biochemical effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds on the central nervous system. Int J Biochem Cell Biol 27:443-455.

Uno S, Dalton TP, Sinclair PR, Gorman N, Wang B, Smith AG, Miller ML, Shertzer HG, Nebert DW (2004) Cyp1a1(-/-) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyria. Toxicol Appl Pharmacol 196:410-421.

Vaglio P, Lamesch P, Reboul J, Rual JF, Martinez M, Hill D, Vidal M (2003) WorfDB: the Caenorhabditis elegans ORFeome Database. Nucleic Acids Res 31:237-240.

Vorderstrasse BA, Steppan LB, Silverstone AE, Kerkvliet NI (2001) Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. Toxicol Appl Pharmacol 171:157-164.

Walisser JA, Bunger MK, Glover E, Bradfield CA (2004) Gestational exposure of Ahr and Arnt hypomorphs to dioxin rescues vascular development. Proc Natl Acad Sci U S A 101:16677-16682.

Walisser JA, Glover E, Pande K, Liss AL, Bradfield CA (2005) Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types. Proc Natl Acad Sci U S A 102:17858-17863.

White JG, Southgate E, Thomson JN, Brenner S (1976) The structure of the ventral nerve cord of Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci 275:327-348.

White JG, Southgate E, Thomson JN, Brenner S (1986) The structure of the nervous system of the nematode C. elegans. Philos Trans R Soc Lond B Biol Sci 314:1-340.

Whitlock JP, Jr. (1999) Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 39:103-125.

Wood WB (1988) The Nematode Caenorhabditis elegans. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Xu C, Li CY, Kong AN (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res 28:249-268.

CHAPTER 2

The *C. elegans* aryl hydrocarbon receptor, AHR-1, regulates neuronal development.

A paper published in

Developmental Biology, 2004, 270(1): 64-75.

Hongtao Qin[1] and Jo Anne Powell-Coffman[2]

ABSTRACT

The mammalian aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the toxic effects of dioxins and related compounds. Dioxins have been shown to cause a range of neurological defects, but the role of AHR during normal neuronal development is not known. Here we investigate the developmental functions of *ahr-1*, the *C. elegans* aryl hydrocarbon receptor homolog. We show that *ahr-1*:GFP is expressed in a subset of neurons, and we demonstrate that animals lacking *ahr-1* function have specific defects in neuronal differentiation, as evidenced by changes in gene expression, aberrant cell migration, axon branching, or supernumerary neuronal processes. In *ahr-1* deficient animals, the touch receptor neuron AVM and its sister cell, the interneuron SDQR, exhibit cell and axonal migration defects. We show that dorsal migration of SDQR is mediated by UNC-6/Netrin, SAX-3/Robo, and UNC-129/TGF , and this process requires the functions of both *ahr-1* and its transcription factor dimerization partner *aha-1*. We also document a

---

[1] Primary researcher and author
[2] Author for correspondence

role for *ahr-1* during the differentiation of the neurons that contact the pseudocoelomic fluid. In *ahr-1*-deficient animals, these neurons are born, but they do not express the cell type specific markers *gcy-32*:GFP and *npr-1*:GFP at appropriate levels. Additionally, we show that *ahr-1* expression is regulated by the UNC-86 transcription factor. We propose that the AHR-1 transcriptional complex acts in combination with other intrinsic and extracellular factors to direct the differentiation of distinct neuronal subtypes. These data, when considered with the neurotoxic effects of AHR-activating pollutants, support the hypothesis that AHR has an evolutionarily conserved role in neuronal development.

INTRODUCTION

During multicellular development, individual cells integrate instrinsic and extracellular signals as they execute developmental programs. Certain manmade pollutants perturb these processes and are hazards to human health. They can also be keys to deciphering normal developmental programs. The study of dioxin toxicity led to the identification of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, as an important regulator of cell fates. Dioxins and related compounds have been shown to cause birth defects, cancer, hepatotoxicity, or immunological deficiencies in a variety of vertebrates. Most, if not all, of these pathologies are due to inappropriate activation of AHR (Schmidt and Bradfield, 1996; Whitlock, 1999). AHR is expressed in many cell types, including the brain (Abbott et al., 1995; Jain et al., 1998; Petersen et al., 2000). Genetic studies in mice have confirmed that loss of AHR function results in slow growth and reduced fertility (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt et al., 1996). However, the cellular and developmental requirements for AHR in most tissues are not understood.

AHR and its nuclear dimerization partner, ARNT, are both members of a family of transcription factors that contain an N-terminal basic-helix-loop-helix (bHLH) DNA-binding and dimerization motif, and a PAS domain, named for its discovery in *Drosophila* Period, mammalian ARNT, and *Drosophila* Singleminded (Gu et al., 2000). In the absence of ligand, AHR resides in the cytoplasm in a complex with 90 kDa heat shock proteins (HSP90) and additional chaperonins (Carver and Bradfield, 1997; Denis et al., 1988; Ma and Whitlock, 1997; Meyer et al., 2000; Perdew, 1988). Upon binding ligand, the receptor translocates to the nucleus, dissociates from HSP90, and binds ARNT (Probst et al., 1993; Reyes et al., 1992). The AHR:ARNT complex binds to specific DNA sequences to regulate the expression of target genes (Hankinson, 1995).

The most potent known AHR agonist is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD can cause birth defects, cancer, hepatotoxicity, immunological deficiencies, or death

(Schmidt and Bradfield, 1996). Fetal exposure to TCDD can cause a range of neurological defects in humans and other primates, as evidenced by reduced cognitive abilities and altered reproductive behaviors (Patandin et al., 1999; Peterson et al., 1993; Schantz and Bowman, 1989). AHR-activating ligands have been shown to cause abnormal cell death in the zebrafish brain and asymmetric brain development in the chick (Dong et al., 2002; Henshel et al., 1997). TCDD exposure also minimizes sex-specific differences in gene expression in certain rat GABAergic neurons (Hays et al., 2002). These toxicological studies suggest that inappropriate activation of AHR can alter cell fates in the developing nervous system.

Multiple laboratories have disrupted the mouse AHR gene. As expected, AHR-deficient mice are resistant to many of the deleterious effects of AHR-activating pollutants (Fernandez-Salguero et al., 1995; Matikainen et al., 2001; Mimura et al., 1997; Schmidt et al., 1996; Shimizu et al., 2000; Vorderstrasse et al., 2001). All of the AHR-deficient strains show reduced growth, reproduction, and survival. Additionally, defects have been reported in the liver, heart, ovary, and the vascular and immune systems (Benedict et al., 2000; Fernandez-Salguero et al., 1995; Lahvis et al., 2000; Schmidt et al., 1996). These analyses suggest that AHR has important developmental functions that may be instructed by endogenous ligands. This hypothesis is further supported by the recent identification and biochemical characterization of an AHR-activating ligand in lung (Song et al., 2002).

We have investigated AHR function and regulation in a genetic model system, the nematode *C. elegans*. The *C. elegans* orthologs of AHR and ARNT are encoded by the *ahr-1* and *aha-1* genes (which encode the AHR-1 and AHA-1 proteins) (Powell-Coffman et al., 1998). Here, we demonstrate that the functions of *C. elegans ahr-1* and *aha-1* are required to direct the differentiation of specific neurons. We describe requirements for *ahr-1* during the migration and differentiation of AVM and SDQR, two neurons in the Q lineage. Addtionally, we present evidence that AHR-1 acts downstream of UNC-86 in a

transcriptional cascade that activates expression of the *gcy-32* soluble guanylyl cyclase and the *npr-1* neuropeptide receptor in the neurons that mediate social feeding.

Materials and Methods

C. elegans culture and genetic analyses

C. elegans were cultured using standard methods (Brenner, 1974). The wild-type parent for most strains used in this study was *C. elegans* var. Bristol strain N2. The following mutant alleles were used in this study: LGI: *ahr-1 (ia03), aha-1 (ia01), unc-40 (n473), unc-40 (e1430)*; LGIII: *unc-86 (n306), unc-86 (n846)*; LGIV: *unc-5 (e53), unc-129 (ev557);* LGX: *npr-1 (n1335), sax-3 (ky123), unc-6 (ev400).*

Previously published strains carrying cell-type-specific GFP reporters were also used in this study. They include NW1229 [*F25B3.3::GFP(evIs111)*] , TU2589 [*mec-18::gfp(uIs25)V*], and DA1295 [*gcy-32::gfp(adEX1295)*] (Yu et al., 1997; Altun-Glutekin et al., 2001; Wu et al., 2001).

To isolate *ahr-1 (ia03)* mutations, N2 worms were treated with ethyl methansulfonate and allowed to self-fertilize. Polymerase chain reaction (PCR) was used to detect *ahr-1* deletions in the descendents of the mutagenized animals (Jansen et al., 1997). The *ia03* allele was backcrossed to wild type seven times before any phenotypic analyses were performed. RT-PCR was used to amplify cDNA fragments from *ia03* homozygotes. A cDNA was detected in which exon 3 was spliced to exon 8, resulting in a frameshift and an early translational stop.

Construction of reporter genes

The pJ360 *ahr-1::gfp* plasmid was constructed as follows: A 15,579 bp HindIII genomic fragment containing the entire *ahr-1* coding sequence and 3287 bp of sequence 5' to the translational start codon was ligated into the HindIII site of T7/T3 18, creating pJ301. To remove part of the polylinker sequence, this vector was cut with SalI and SmaI, treated with Klenow, and religated. The resulting construct was cut with BstXI (codon 496, 3' to the PAS domain), and a linker containing a XmaI site was added (5'

TAGCCCGGGGTCGACAGC) to create pJ356. The AgeI-XmaI GFP-encoding fragment from pPD95.02 was ligated into the XmaI site of pJ356 to generate the pJ360 *ahr-1:gfp* fusion construct. To make the pHT102 *ahr-1::gfp* plasmid, a 5377bp HindIII - BamHI genomic fragment, including over 3 kb of sequence 5' to the translational start codon, exon1, intron 1, and part of exon 2, was ligated into the HindIII and BamHI sites of the pPD95.75 GFP expression vector. A. Fire (Carnegie Institute) kindly provided the vectors containing GFP cassettes.

Transgenic strains carrying *ahr-1::gfp* constructs were obtained by microinjecting plasmid DNA into the germline of N2 animals following standard procedures. The *rol-6* plasmid pRF4 was used as the co-injection marker in each case (Mello et al., 1991). The strains analyzed for this study include: ZG93: *ia*Is2 (pJ360) and ZG102: *ia*Ex102 (pHT102).

Identification of GFP-expressing cells and phenotypic analyses

C. elegans were mounted on 4% agarose pads in M9 buffer containing 10 mM sodium azide, and observed by DIC microscopy or fluorescence microscopy on a Nikon E800 microscope connected to a Spot RT digital camera (Roche Diagnostics). For some figures, images were captured on an inverted Nikon Eclipse microscope connected to a Prairie Technologies (Middleton, WI) Scanning Laser Confocal Microscope controlled by Prairie Technologies software. Images were analyzed and reconstructed into a single plane for presentation using Metamorph software (Universal Imaging, West Chester, PA). Cells were identified according to the size and the shape of their nuclei, the morphology of their processes, and their absolute and relative positions. The *gcy-32::gfp* and *mec-18::gfp* markers were used to confirm the identity of some neurons (Yu et al., 1997; Wu et al., 2001). The positions of AVM and PLM in various mutant backgrounds were assayed in animals expressing the *mec-18:gfp* marker.

SDQR cell position was scored in animals carrying the *F25B3.3::gfp* panneural marker (Altun-Gultekin et al., 2001). Dorsal migration of the SDQR cell was scored as defective if SDQR was ventral to the ALMR-associated axon. If the position of SDQR was close to that of AVM, then we used two criteria to distinguish between the two cells. First, if only one of the two cells extended an axon ventrally to the ventral nerve cord, then that cell was scored as AVM. Second, if other characteristics did not distinguish the two cells, then the cell body with the more dorsal and anterior position was scored as SDQR. The anterior-posterior positions of SDQR and AVM relative to Vn.a and Vn.p epidermal cells were scored between 13 and 15 hours after hatching. To study GFP fluorescence in the URX, AQR, and PQR cells, the arrays in the ZG102 and DA1295 strains were crossed into the *ahr-1*- or *unc-86*-deficient genetic backgrounds.

Social feeding assays were performed as previously described (de Bono et al., 2002). Eighty worms were scored in each experiment. Each genotype was assayed in 6 independent experiments.

RESULTS

ahr-1:GFP expression in a subset of neurons

To identify cells that express AHR-1, we made reporter genes in which the expression of GFP-tagged fusion proteins are directed by *ahr-1* regulatory sequences. The pJ360 construct includes the entire *ahr-1* genomic sequence (Fig. 1A), and transgenic animals express this fusion protein in a subset of neuronal nuclei. Expression from pJ360 rescues specific defects in *ahr-1*-deficient animals (see below for details). Nuclear localization of this GFP-tagged AHR-1 protein indicates that AHR-1 is in the appropriate subcellular localization to be a transcriptional regulator. To facilitate the identification of fluorescing cells, we constructed a second reporter. The pHT102 transgene lacks most of the *ahr-1* coding sequence and labels axons as well as nuclei (Fig. 1). Both *ahr-1*:GFP reporters are expressed during embryonic and larval development. Expression is first detected in 2 cells 260 minutes after the first cleavage. By midembryogenesis (pre-comma stage), 14 cells express the pJ360 *ahr-1*:GFP fusion gene. At the two-fold stage of embryogenesis, two cells express *ahr-1*:GFP in the tail, and the remaining fluorescing cells are in the forming head. We have identified all of the cells that detectably express pJ360 during the first larval stage. *ahr-1*:GFP is expressed in twenty-eight neurons, several blast cells, and two phasmid socket cells. The neurons that express *ahr-1*:GFP include ALNR / ALNL, AQR / PQR, AVM / PVM, BDUR / BDUL, PLMR / PLML, PLNR / PLNL, PHCL/PHCR, PVWL/PVWR, RMEL/RMER, SDQR / SDQL, and URXR / URXL. (With few exceptions, the *C. elegans* nervous system is bilaterally symmetrical. For example, ALNR and ALNL are the right and left ALN neurons, respectively.) These neurons belong to multiple neuronal subtypes, including touch receptor cells, interneurons, and neurons that contact pseudocoelomic fluid. The T.pa, T.ppa, and T.ppp blast cells in the tail express *ahr-1*:GFP, as do all of their descendents, including the PHso1 and PHso2 phasmid socket cells. *ahr-1*:GFP is also expressed in the MI and I3 neurons in the pharynx and the G2 and

W blast cells. Four additional cells in the head express *ahr-1*:GFP, and we have tentatively identified them as the ASK and RIP neurons.

Six of the *ahr-1*:GFP-expressing neurons arise from the Q neuroblasts. At hatching, *C. elegans* larvae contain 2 Q neuroblasts: QR (on the right side) and QL (on the left side). During the first larval stage, QR migrates anteriorly, and QL migrates posteriorly. We did not detect *ahr-1*:GFP expression in QR or QL, but the *ahr-1*:GFP transgenes are expressed in all of the descendents of these neuroblasts during migration and differentiation. In most animals, expression of *ahr-1*:GFP is detectable before the QR daughters migrate to a position anterior to the V4 hypodermal precursor cell. (The migration of QR descendents is diagrammed in Figure 2A.) QR descendents form the AQR, SDQR, and AVM neurons (Fig 2A). QL gives rise to the PQR, SDQL, and PVM neurons (Sulston and Horvitz, 1977). Each neuron adopts a distinct cell fate, characterized by cellular location, axon projection, or cell-type-specific gene expression.

Neuronal requirements for AHR-1 and its transcriptional partner, AHA-1

To examine the role(s) of *ahr-1* during neuronal development, we generated a library of mutagenized *C. elegans* and identified a strain carrying a 1517 bp deletion in the *ahr-1* gene. This mutation removes sequence from 205 bp 5' of exon 4 to 30 bp 5' of exon 8, including most of the PAS domain, and it introduces a premature translational stop codon (Fig. 2B). It is likely a strong loss-of-function allele. In support of this, *C. elegans* carrying the *ahr-1 (ia03)* deletion in trans to *mnDf111*, a large deficiency in the region, have SDQR defects similar to those found in *ahr-1 (ia03)* homozygotes (see below). Animals that are homozygous for the *ahr-1 (ia03)* mutation are viable and exhibit subtle locomotive defects. They do not have dramatic defects in life span, fecundity, or dauer formation.

To characterize the cellular requirements for *ahr-1*, we introduced cell-type-specific markers into *ahr-1 (ia03)* mutants. We observed cell and axonal pathfinding defects in the AVM and SDQR neurons. SDQR and AVM are sister cells that arise from the division of

QR.pa (Fig 2A). SDQR migrates dorsally and anteriorly to a position on the axon tract associated with ALMR and ALNR. The SDQR interneuron then extends a single axon to the dorsal sublateral nerve (Fig. 1D). AVM migrates ventrally and anteriorly, and it extends a single axon to the ventral nerve cord (Figs. 1C, 2A). AVM and SDQR are often displaced posteriorly in *ahr-1 (ia03)* mutants (Figs. 2C, D). The *ahr-1 (ia03)* animals also exhibit a range of defects in AVM axonal pathfinding and morphology. In 5% of the 211 mutant worms examined, the AVM cell extended 2 neuronal processes, and in each case, one of the processes was directed posteriorly. In 11% of the AVM cells that projected a single axon from the cell body, the initial extension was directed posteriorly, rather than ventrally. In 7% of the *ahr-1 (ia03)* animals, the AVM axon branched, a defect that was very rare in wild-type animals (0.6%; n = 180). These data indicate that *ahr-1* function is required to direct AVM differentiation and to instruct the response of the AVM cell and axon to external guidance cues.

ahr-1 also has a role in the development of the PLM neurons. Like AVM and PVM, the PLM neurons are touch receptor cells, and they express the *mec-18*:GFP marker (Wu et al., 2001). In wild-type animals, the PLMR and PLML cell bodies are located at the posterior edge of the lumbar ganglia in the tail, and several nuclei are visible between either PLM cell body and the anus (100%; n = 91; Fig. 3A). Each PLM neuron extends a posterior dendritic process and an anteriorly directed axon (White et al., 1986). In 35% of *ahr-1 (ia03)* animals examined, at least one of the PLM cell bodies was displaced to a position as anterior as the anus (Fig 3C,D). In some cases, an additional *mec-18*:GFP-expressing "PLM cell" was present (Fig 3E,F). Although this phenotype was rare (4 cases in 120 animals), it is notable, since it indicates that loss of *ahr-1* function may cause lineage defects or disrupt programmed cell death.

Defects in SDQR cell and axonal migration are highly penetrant in *ahr-1*-defective animals. In 53% of *ahr-1 (ia03)* mutants, SDQR fails to migrate dorsally to the ALMR-associated nerve (Fig 4B, D, E). Although some cells migrate to the appropriate

dorsal-ventral position, 91% of those SDQR neurons exhibit axonal pathfinding defects. In *ahr-1 (ia03)* mutants, the SDQR axon did not "wander" randomly. Instead, it usually projected anteriorly. In many cases, the initial projection was dorsal or ventral, but the axon would usually associate with a lateral nerve and turn anteriorly. The expression of AHR-1:GFP by the pJ360 transgene restores dorsal guidance of SDQR in *ahr-1 (ia03)* animals (Fig. 4E). The mutant phenotype is also rescued by an fusion of *ahr-1* genomic and cDNA sequences that lacks introns 3 – 10 (data not shown).

In many *ahr-1*- or *aha-1*-deficient animals, SDQR migrates to a position close to that of its sister, AVM. In some cases, axons from both cells project anteriorly, rather than dorsally or ventrally (for example, see Figs. 4B, D). We examined the expression of *mec*-18:GFP, which is normally expressed in AVM, but not SDQR (Wu et al. 2001). The normal *mec-18*:GFP expression pattern is maintained in *ahr-1* mutant animals. This shows that SDQR and AVM adopt distinct fates in the absence of *ahr-1* function.

AHR-1 is known to bind DNA as a heterodimer with the AHA-1 bHLH-PAS transcription factor *in vitro* (Powell-Coffman et al., 1998). To further test the model that a AHR-1:AHA-1 DNA-binding complex directs neuronal development, we examined SDQR migration in *aha-1*-deficient animals. *aha-1 (ia01)* is a predicted null allele, and *C. elegans* that are homozygous for the *ia01* mutation arrest development as young larvae. Viability can be restored by the pHJ32 transgene, which directs expression of *aha-1* in most non-neuronal pharyngeal cells (Jiang, Wu, and Powell-Coffman, manuscript in preparation.) *aha-1 (ia01)* arrested larvae and *aha-1 (ia01)* mutants rescued by the pHJ32 transgene exhibit defects in SDQR dorsal-ventral pathfinding (Fig. 4E). Thus, SDQR differentiation, as assayed by cellular migration, requires the functions of both *ahr-1* and *aha-1*. This represents genetic evidence that AHR-1 and AHA-1 interact to regulate SDQR development.

Genetic requirements for dorsal-ventral guidance of SDQR

Prior studies have shown that dorsal migration of the SDQR cell and axon is mediated by UNC-6/netrin and the UNC-5 and UNC-40 netrin receptors. SDQR pathfinding defects in *unc-6* mutants are not fully penetrant, and this has suggested that extracellular signals other than netrin direct SDQR migration (Kim et al., 1999). We noted similarities between the SDQR cell migration phenotypes in *ahr-1 (ia03)* animals and in *unc-6* loss-of-function mutants. This raised the possibility that the AHR-1:AHA-1 transcriptional complex might modulate response to the UNC-6/ netrin signal. A nonexclusive alternative is that AHR-1 mediates cellular responses to one or more other pathfinding signals. The SLT-1/Slit and UNC-129/TGF guidance cues have also been shown to mediate dorsal-ventral pathfinding in *C. elegans,* but their roles in SDQR migration have not been assessed (Colavita et al., 1998; Hao et al., 2001; Zallen et al., 1998). To more fully understand dorsal-ventral guidance of SDQR and the role of AHR-1 in this process, we compared the *ahr-1 (ia03)* mutant phenotype to those in animals deficient in *unc-5, unc-6, unc-40, sax-3,* or *unc-129.* The migration defects we observed in *unc-5*, *unc-6*, or *unc-40* mutants (Fig. 5) are in agreement with previously published analyses (Kim et al., 1999). In animals lacking *unc-129* function, SDQR failed to migrate to a dorsal position in 34% of the animals assayed (Fig. 5A; n = 130). *sax-3*, which encodes the receptor for SLT-1, also mediates SDQR cell migration. SDQR migrated to a position ventral to ALMR in 17% of *sax-3*-defective animals (n = 203). Double mutant analyses indicate that *ahr-1 (ia03)* enhances the SDQR cell migration defects caused by presumed null mutations in *unc-5*, *unc-6*, *unc-40, sax-3,* or *unc-129* (Fig. 5; $p < 0.008$).

We also examined axon migration patterns. Since the milieu of extracellular guidance cues changes along the dorsal-ventral axis of the worm, we distinguished between projections from dorsally- and ventrally-located SDQR cells. In the absence of *ahr-1* function, dorsally-located SDQR cells usually project a single axon anteriorly, and dorsal axon migration is rare (Fig 5B). By this measure, the axon migration phenotype of *ahr-1 (ia03)* animals differs from that of the other mutants examined (Fig. 5B). The axon of a

ventrally located SDQR cell projects dorsally 56% of the time in an *ahr-1*-deficient animal. This is another distinction between *ahr-1 (ia03)* mutants and animals lacking *unc-5, unc-6* or *unc-40* function. Most ventrally located SDQR cells in *unc-5*-, *unc-6*-, or *unc-40*-deficient animals have ventral axon projections (Fig 5C). Axon migration defects in *sax-3 (ky123)* or *unc-129 (ev557)* mutants are less severe than those in *ahr-1 (ia03)*.

AVM axon migration defects are enhanced by the *ahr-1 (ia03)* mutation in an *unc-6* mutant background. Double mutants lacking *unc-6* and *ahr-1* function exhibit more extensive branching of neuronal processes than either single mutant (data not shown). Collectively, these data indicate that *ahr-1* has functions in SDQR and AVM that are independent of Netrin signaling.

ahr-1 regulates a subset of the URX, AQR, and PQR differentiation program

The *ahr-1*:GFP-expressing cells AQR, PQR, URXL, and URXR are an interesting subclass of neurons. They directly contact the pseudocoelomic fluid in the body cavity, and they have been shown to have a role in social feeding behavior (Coates and de Bono, 2002). In the wild, some *C. elegans* feed in groups, while other strains are solitary feeders (Hodgkin and Doniach, 1997). This behavioral trait is regulated by NPR-1, a G-protein-coupled neuropeptide receptor (de Bono and Bargmann, 1998). Animals carrying a strong loss-of-function allele of *npr-1* clump together on the bacterial food source. Solitary feeding behavior can be restored by expression of wild type NPR-1 in AQR, PQR, and URX (Coates and de Bono, 2002). Using a panneural marker and *ahr-1*:GFP, we determined that AQR, PQR, and URX are formed in *ahr-1 (ia03)* animals. We examined two markers, *gcy-32*:GFP and *npr-1*:GFP, to assess the role of *ahr-1* in the differentiation of this neuronal subclass. *gcy-32* encodes a soluble guanylyl cyclase gene. In wild-type animals, *gcy-32*:GFP strongly and specifically labels the AQR, PQR, and URX neurons (Yu et al., 1997). As shown in Tables 1 and 2, *ahr-1*-defective animals do not express *gcy-32*:GFP at high levels.

We compared the expression of a *npr-1*:GFP translational fusion (Coates and de Bono, 2002) in wild-type and *ahr-1*-deficient animals. In a wild-type genetic background, the *npr-1*:GFP translational fusion is detectable in 84% of URX cells (n = 134). In *ahr-1 (ia03)* animals, *npr-1*:GFP expression is detectable in only 3.6% of URX cells (n = 112). Expression of *npr-1*:GFP is also diminished in AQR and PQR.

To further assess the differentiated state of AQR, PQR, and URX in *ahr-1 (ia03)* animals, we compared the *ahr-1* phenotype to the effects of *egl-2*-mediated inactivation of these neurons. Certain gain-of-function mutations in the EGL-2 potassium channel cause the channel to open at inappropriate voltages, thereby dramatically compromising neuronal function (Weinshenker et al., 1999). Coates and de Bono (2002) have shown that expression of *egl-2 (gf)* in AQR, PQR, and URX almost completely suppresses social feeding behavior in *npr-1*-deficient animals. We reasoned that if a loss-of-function mutation in *ahr-1* resulted in decreased *npr-1* expression but did not dramatically inhibit AQR, PQR, URX differentiation, then *ahr-1 (ia03)* animals would be expected to feed in groups. However, if deletion of *ahr-1* were equivalent to genetic inactivation of this neuronal subclass, then *ahr-1 (ia03)* would suppress *npr-1*-mediated social feeding behavior. We report that only 1.9% of *ahr-1 (ia03)* animals feed in groups, while 53% of *npr-1 (n1353)* animals form groups on the bacterial food source. Further, *npr-1*-mediated clumping on food is suppressed by mutation of *ahr-1*: 24 % of *ahr-1 (ia03); npr-1 (n1353)* double mutants feed in groups. These data suggest that AQR, PQR, and URX have decreased function in *ahr-1 (ia03)* animals, but are not completely inactivated.

Regulation of *ahr-1* expression by UNC-86

UNC-86 is a POU-domain-containing transcription factor that is required for the specification of certain neuroblast lineages and neuronal cell fates (Finney and Ruvkun, 1990). Three lines of evidence suggested to us that AHR-1 might act downstream of the UNC-86 transcription factor to regulate the differentiation of neuronal subtypes. First, the

ahr-1:GFP-expressing cells that we have identified here include a subset of the 57 post-mitotic neurons that express UNC-86. Second, appropriate differentiation of many of these neurons requires the functions of both UNC-86 and AHR-1. The *unc-86*-defective phenotype is much more severe than that seen in *ahr-1* mutants. For example, in animals lacking *unc-86* function, QR.p and QL.p reiterate the fates of their respective mother cells. As a consequence, additional cells are formed with AQR / PQR characteristics, and AVM, PVM, SDQR, and SDQL are not born (Finney and Ruvkun, 1990). These loss-of-function phenotypes suggest that, if the two proteins function in a regulatory cascade to direct neuronal development, UNC-86 acts upstream of AHR-1. Third, in animals that lack *unc-86* function, the URX cells are present, but they fail to express the *gcy-32*:GFP marker (Rohrig et al., 2000). As shown in Table 2, expression of *gcy-32*:GFP also requires *ahr-1*.

To explore the regulatory relationship between UNC-86 and AHR-1, we examined the expression of *ahr-1*:GFP in *unc-86* mutants, and we found that high levels of *ahr-1*:GFP expression in URX require *unc-86* function (Table 2). Thus, in URX, UNC-86 regulates *ahr-1* expression, and AHR-1 positively regulates the expression of *gcy-32*:GFP and *npr-1*:GFP.

To determine whether *ahr-1* expression is autoregulated, we compared expression of the *ahr-1*:GFP reporter in wild type and *ahr-1 (ia03)* animals. We observed that *ahr-1*:GFP was expressed at slightly lower levels in the absence of *ahr-1* function. As shown in Table 2, in URX, the *ahr-1 (ia03)* mutation has less impact on *ahr-1*:GFP expression than do the *unc-86* strong loss-of-function mutations.

DISCUSSION

Prior studies have shown that dioxins and other compounds that inappropriately activate AHR are neurotoxic to a range of vertebrate species, including humans (Dong et al., 2002; Hays et al., 2002; Henshel et al., 1997; Patandin et al., 1999). Here, we demonstrate that *C. elegans* AHR-1 has important roles in normal neuronal development. *ahr-1*:GFP reporters are expressed in a subset of neurons, and *C. elegans* containing a deletion in the *ahr-1* gene have specific defects in neuronal differentiation, including altered morphology, failure to express cell-type-specific markers (i.e. *gcy-32*:GFP and *npr-1*:GFP), or defects in cell and axonal migration. These data suggest that an ancestral AHR gene regulated neuronal development and that some of these functions may have been evolutionarily conserved.

Role of AHR-1 in neuronal development

The nervous system of an adult *C. elegans* hermaphrodite consists of 302 neurons, including at least 118 neuronal subtypes (White et al., 1986). Each individual neuron is defined by its developmental history, cell and axonal morphology, synaptic connections, and gene expression pattern. Mutational studies have demonstrated that transcription factors play key roles during the specification and differentiation of neuronal cell fates in *C. elegans* (Ruvkun, 1997; Hallam et al., 2000; Sarafi-Reinach et al., 2001; Altun-Gultekin et al., 2001). The molecular bases of neural subtypes have also been studied intensively in Drosophila and vertebrate central nervous systems, and current models suggest that neuronal cell fates are defined by the expression and activation of unique combinations of transcription factors (Jurata et al., 2000; Shirasaki and Pfaff, 2002). The data presented here suggest that in *C. elegans*, the AHR-1 transcriptional complex has critical roles in the differentiation programs of several neurons belonging to multiple subtypes.

We propose that *ahr-1* function is required for a subset of the URX, AQR, and PQR differentiation programs. These neurons contact the psuedocoelomic fluid and regulate social feeding behavior. In these cells, AHR-1 acts downstream of UNC-86 to activate the expression of at least two cell-type-specific markers: *gcy-32*:GFP and *npr-1*:GFP. Genetic inactivation of URX, AQR, and PQR almost completely suppresses *npr-1*-mediated clumping behavior (Coates and de Bono, 2002). In contrast, the *ahr-1 (ia03)* mutation only partially suppresses this behavior.

The AVM and PLMR/L touch receptor cells also develop aberrantly in *ahr-1 (ia03)* mutants, as evidenced by cell and axonal migration defects in AVM and mispositioning of PLM. Expression of the touch cell-specific markers *mec-18*:GFP and *mec-7*:GFP is not disrupted in *ahr-1 (ia03)* mutants. This suggests that AHR-1 acts in concert with other intrinsic and extracellular factors to execute the AVM and PLM developmental programs. The presence of an "extra PLM cell" in some mutants is very intriguing. This phenotype suggests at least two hypotheses. Two close lineal relatives of PLM undergo programmed cell deaths. *ahr-1* function might be required to efficiently implement these deaths. This model is consistent with other studies that have shown that AHR-activating ligands can induce the death of mouse ovarian cells or zebrafish brain cells (Dong et al., 2002; Matikainen et al., 2001). Alternatively, loss of *ahr-1* may cause another cell, such as ALN, to inappropriately adopt the PLM fate. Since the "extra PLM cell" phenotype is relatively rare, it will be challenging to distinguish between these models by lineage studies.

AHR-1:GFP is also expressed in RMEL and RMER. The RME motor neurons control head movements and express the neurotransmitter GABA. X. Huang and Y. Jin isolated another loss-of-function allele of *ahr-1* in a screen for mutations that alter RME cell fates. In collaboration with our research group, they have shown that in the absence of *ahr-1* or *aha-1* function, RMEL and RMER adopt fates similar to those of RMEV and RMED. Further, ectopic expression of *ahr-1* in RMEV and RMED can transform these

cells into RMEL/V-like neurons (Huang et al., 2004). This strengthens the model that AHR-1 and AHA-1 interact to regulate neuronal cell fate specification.

Regulation of AHR-1 expression by the UNC-86 transcription factor

In some cells, *ahr-1* is regulated by the UNC-86 transcription factor. *ahr-1*-deficient worms exhibit defects in AVM, AQR, SDQR, PLMR/L, PQR, and URXR/L. The identity of each of these neurons is also instructed by *unc-86* (Finney and Ruvkun, 1990; Rohrig et al., 2000). The defects in *unc-86*-deficient animals are more severe than those in *ahr-1 (ia03)* mutants. This suggests that if UNC-86 and AHR-1 function in a common pathway, AHR-1 is downstream of UNC-86.

We tested the hypothesis that *ahr-1* expression in URX is regulated by UNC-86 by comparing *ahr-1*:GFP fluorescence in wild type and *unc-86*-deficient animals. We found that normal levels of *ahr-1*:GFP expression in these cells requires *unc-86* function. Moreover, *C. elegans* carrying null mutations in *ahr-1* or *unc-86* fail to express the *gcy-32*:GFP reporter at wild-type levels. Interestingly, sequences 5' to the *ahr-1* translational start include consensus UNC-86 binding sites (5' CATnnnA/TAAT), while the *C. elegans* genomic sequences in the *gcy-32*:GFP reporter do not. The *gcy-32*:GFP regulatory region does contain two possible binding sites for the AHR-1 transcriptional complex (5' KnGCGTG). Collectively, these data support a model in which UNC-86 acts upstream of AHR-1 in a transcriptional cascade to activate *gcy-32* expression in URX.

Recent studies indicate that expression of *ahr-1* mRNA expression is negatively regulated by the MEC-3 transcription factor in touch receptor cells (Zhang et al., 2002). MEC-3 is an important regulator of touch cell fate, and it interacts with UNC-86 to regulate gene expression (Duggan et al., 1998; Way and Chalfie, 1988; Xue et al., 1993). Chalfie and colleagues recently examined MEC-3-dependent gene expression in cell populations highly enriched for ALMR/L and PLMR/L touch receptor neurons. They found *ahr-1* mRNA was expressed at a 2-fold higher level in *mec-3* mutants relative to wild-type *C.*

elegans (Zhang et al., 2002). We compared the expression of *ahr-1*:GFP in wild-type and *mec-3*-deficient animals, and did not observe any dramatic differences. This is not inconsistent with the 2-fold difference reported in the microarray studies. We note that the first intron of *ahr-1* contains a predicted binding site for the UNC-86:MEC-3 complex (5' CATCGAAATCCATTG; bases identical to the UNC-86:MEC-3 binding site in *mec-3* are underlined). These findings suggest that MEC-3 and UNC-86 may act cooperatively to repress *ahr-1* transcription in cells such as PLMR/L.

SDQR migration

Prior studies had demonstrated that dorsal-ventral guidance of SDQR is directed by UNC-6/ Netrin (Kim et al., 1999). Our data indicate that at least two other pathways direct SDQR cell and axon migration. Loss-of-function mutations in either the *sax-3*/Robo receptor or the *unc-129* ligand result in failure of SDQR to migrate to its appropriate dorsal position. This defect is less penetrant in *sax-3* or *unc-129* mutants than in *unc-6*-deficient animals, and this indicates that SDQR is more reliant on the UNC-6/Netrin guidance cue than on the other two pathways.

ahr-1 (ia03) animals have highly penetrant SDQR cell and axonal migration defects. Interestingly, SDQR axon migration defects in *ahr-1 (ia03)* animals appear to be distinct from that of the other mutants tested, and the cell migration defects caused by predicted null mutations in *unc-5, unc-6, unc-40* are enhanced by the *ahr-1 (ia03)* deletion mutation. *aha-1,* the gene that encodes the AHR-1 transcriptional dimerization partner, is also required to direct migration of the SDQR cell. These data suggest a model in which the AHR-1:AHA-1 complex regulates a fraction of the gene expression pattern that defines SDQR, and animals deficient in *ahr-1* or *aha-1* function may respond inappropriately to developmental signals.

Other studies have shown that cell-type-specific responses to the UNC-6/Netrin and SLT-1/Slit signals are dependent upon networks of interacting proteins and intracellular

second messengers (Hao et al., 2001; Huang et al., 2002; Yu et al., 2002). Mutation of the UNC-2 or EGL-19 voltage-gated calcium channels has been shown to cause mispositioning of SDQR and AVM (Tam et al., 2000). A full understanding of how the AHR-1 transcriptional complex influences the differentiation of SDQR will ultimately require the identification of AHR-1 target genes and characterization of their respective functions.

Comparative analyses of AHR regulation and function

Studies of AHR in evolutionarily divergent model systems will likely elucidate the ancestral functions of AHR, and they may identify conserved AHR interacting proteins. AHR homologs from *C. elegans*, *Drosophila melanogaster* and soft-shell clam share compelling molecular and biochemical similarities to mammalian AHR. These proteins contain basic-helix-loop-helix and PAS motifs, dimerize with ARNT, and bind the mammalian xenobiotic response element in a sequence-specific manner (Powell-Coffman et al., 1998; Emmons et al., 1999; Butler et al., 2001). Interestingly, none of these invertebrate AHR proteins bind radiolabeled derivatives of TCDD or -naphthoflavone (Powell-Coffman et al., 1998; Butler et al., 2001). Consistent with these findings, TCDD has no dramatic effect on *C. elegans* development (unpublished observation). This raises the possibility that invertebrate AHR homologs may not be regulated by ligand binding. This hypothesis suggests that the ancestral AHR may have been dedicated to a developmental program, and invertebrate AHR proteins may not have a role in xenobiotic metabolism. Alternatively, the invertebrate AHRs may be activated by a range of ligands that does not include TCDD or beta-naphthoflavone.

Two experimental approaches have been used to address this question. In Drosophila, researchers expressed spineless, the AHR homolog, ectopically. This resulted in nuclear localization of Tango, the ARNT homolog, and phenotypic abnormalities (Duncan et al., 1998; Emmons et al., 1999). These data suggest that activation of Drosophila AHR does not require a spatially restricted ligand. Other Drosophila and *C. elegans* bHLH-PAS

proteins have been shown to localize to the nucleus more efficiently when co-expressed with a dimerization partner (Ward, 1998; Jiang et. al., 2001). Co-expression of Drosophila AHR and ARNT may be sufficient to form an active transcriptional complex (Crews and Fan, 1999).

Yeast expression studies indicate that *C. elegans* AHR is not constitutively active. The PAS domains, which mediate binding to HSP90 and ligand in mammalian AHR, exert a repressive function that inhibits nuclear translocation or transcriptional activation in these assays (Powell-Coffman et al., 1998). No activating ligand has been found, and it is possible that AHR-1 activity is regulated by other signaling pathways, which could covalently modify AHR-1 by phosphorylation or by other means. The activity of mammalian AHR appears to be modulated by multiple mechanisms, including ligand binding, phosphorylation, and interaction with co-activators (Whitlock, 1999; Gu et al., 2000). Some of these regulatory mechanisms likely have ancient origins, while others may be specific to vertebrate AHR.

ACKNOWLEDGEMENTS

We are grateful to Dave Pilgrim, Marty Chalfie, Mario de Bono and Andy Fire for providing GFP plasmids and strains; to Clark Coffman, Chuan Shen, and anonymous reviewers for helpful editorial comments; to Xun Huang and Yishi Jin for sharing information prior to publication; and to the *C. elegans* Sequence Consortium for sequence information. Several *C. elegans* strains were provided by the Caenorhabditis Genetics Center, which is supported by the NIH National Center for Research Resources. Confocal microscopy was performed with the capable assistance of Robert Doyle in the Roy J. Carver Center for Biological Scanning Probe and Laser-based Microscopy at ISU. The pJ360 plasmid was made by J.A.P.-C. as a Leukemia Society Special Fellow in the laboratory of Bill Wood (Boulder, CO). This work was supported by National Science Foundation Award #9874456 to J.A.P.-C.

REFERENCES

Abbott, B. D., Birnbaum, L. S., Perdew, G. H., 1995. Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. Dev. Dyn. 204, 133-43.

Altun-Gultekin Z, Andachi Y, Tsalik EL, Pilgrim D, Kohara Y, Hobert O., 2001. A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans.* Development. 128, 1951-69.

Benedict, J. C., Lin, T. M., Loeffler, I. K., Peterson, R. E., Flaws, J. A., 2000. Physiological role of the aryl hydrocarbon receptor in mouse ovary development. Toxicol. Sci. 56, 382-8.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans.* Genetics 77, 71-94

Butler, R.A., Kelley, M.L., Powell, W.H., Hahn, M.E., Van Beneden, R.J. 2001. An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, Mya arenaria: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone binding. Gene. 278, 223-34.

Carver, L. A., Bradfield, C. A., 1997. Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. J. Biol. Chem. 272, 11452-6.

Coates, J. C., de Bono, M., 2002. Antagonistic pathways in neurons exposed to body fluid regulate social feeding in *Caenorhabditis elegans.* Nature 419, 925-9.

Colavita, A., Krishna, S., Zheng, H., Padgett, R. W., Culotti, J. G., 1998. Pioneer axon guidance by UNC-129, a *C. elegans* TGF-beta. Science 281, 706-9.

Crews, S,T., Fan, C.M., 1999. Remembrance of things PAS: regulation of development by bHLH-PAS proteins. Curr. Opin. Genet. Dev. 9, 580-7.

de Bono, M., Bargmann, C.I., 1998. Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. Cell 94: 679-89

Denis, M., Cuthill, S., Wikstrom, A. C., Poellinger, L., Gustafsson, J. A., 1988. Association of the dioxin receptor with the Mr 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor. Biochem. Biophys. Res. Commun. 155, 801-7.

Dong, W., Teraoka, H., Yamazaki, K., Tsukiyama, S., Imani, S., Imagawa, T., Stegeman, J. J., Peterson, R. E., Hiraga, T., 2002. 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. Toxicol. Sci. 69, 191-201.

Duggan, A., Ma, C., Chalfie, M., 1998. Regulation of touch receptor differentiation by the *Caenorhabditis elegans mec-3* and *unc-86* genes. Development 125, 4107-19.

Duncan, D.M., Burgess, E.A., Duncan, I., 1998. Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev. 12, 1290-303.

Emmons, R.B., Duncan, D., Estes, P.A., Kiefel, P., Mosher, J.T., Sonnenfeld, M., Ward, M.P., Duncan, I., Crews, S.T. 1999. The spineless-aristapedia and tango bHLH-PAS

proteins interact to control antennal and tarsal development in Drosophila. Development 126, 3937-45.

Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., Gonzalez, F. J., 1995. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 268, 722-6.

Finney, M., Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. Cell 63, 895-905.

Gu, Y.-Z., Hogenesch, J. B., Bradfield, C. A. (2000). The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. 40, 519-561.

Hallam, S., Singer, E., Waring, D., Jin, Y. (2000) The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification. Development. 127, 4239-52.

Hankinson, O., 1995. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. 35, 307-40.

Hao, J. C., Yu, T. W., Fujisawa, K., Culotti, J. G., Gengyo-Ando, K., Mitani, S., Moulder, G., Barstead, R., Tessier-Lavigne, M., Bargmann, C. I. 2001. *C. elegans* slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. Neuron 32, 25-38.

Hays, L. E., Carpenter, C. D., Petersen, S. L., 2002. Evidence that GABAergic neurons in the preoptic area of the rat brain are targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin during development. Environ. Health Perspect. 110 Suppl 3, 369-76.

Henshel, D. S., Martin, J. W., DeWitt, J. C., 1997. Brain asymmetry as a potential biomarker for developmental TCDD intoxication: a dose-response study. Environ. Health Perspect. 105, 718-25.

Hodgkin, J., Doniach, T., 1997. Natural variation and copulatory plug formation in *Caenorhabditis elegans*. Genetics 146, 149-64.

Huang, X., Cheng, H. J., Tessier-Lavigne, M., Jin, Y., 2002. MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. Neuron 34, 563-76.

Huang, X., Powell-Coffman, J.A., Jin, Y. 2004. 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*. Development 131, in press.

Jain, S., Maltepe, E., Lu, M. M., Simon, C., Bradfield, C. A., 1998. Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. Mech. Dev. 73, 117-23.

Jansen, G., Hazendonk, E., Thijssen, K. L., Plasterk, R. H., 1997. Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. Nat. Genet. 17, 119-21.

Jiang, H., Guo, R., Powell-Coffman, J.A. 2001. The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc. Natl. Acad. Sci. U S A. 98, 7916-21

Jurata L. W., Thomas, J. B., Pfaff, S. L., 2000. Transcriptional mechanisms in the development of motor control. Curr. Opin. Neurobiol. 10, 72-9.

Kim, S., Ren, X. C., Fox, E., Wadsworth, W. G., 1999. SDQR migrations in *Caenorhabditis elegans* are controlled by multiple guidance cues and changing responses to netrin UNC-6. Development 126, 3881-90.

Lahvis, G. P., Lindell, S. L., Thomas, R. S., McCuskey, R. S., Murphy, C., Glover, E., Bentz, M., Southard, J., Bradfield, C. A., 2000. Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. Proc. Natl. Acad. Sci. U S A 97, 10442-7.

Ma, Q., Whitlock, J. P., Jr., 1997. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Biol. Chem. 272, 8878-84.

Matikainen, T., Perez, G. I., Jurisicova, A., Pru, J. K., Schlezinger, J. J., Ryu, H. Y., Laine, J., Sakai, T., Korsmeyer, S. J., Casper, R. F. et al., 2001. Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. Nat. Genet. 28, 355-60.

Mello C.C., Kramer J.M., Stinchcomb D., Ambros V., 1991. Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959-70.

Meyer, B. K., Petrulis, J. R., Perdew, G. H., 2000. Aryl hydrocarbon (Ah) receptor levels are selectively modulated by hsp90-associated immunophilin homolog XAP2. Cell Stress Chaperones 5, 243-54.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M. et al., 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 2, 645-54.

Patandin, S., Lanting, C. I., Mulder, P. G., Boersma, E. R., Sauer, P. J. and Weisglas-Kuperus, N. 1999. Effects of environmental exposure to polychlorinated biphenyls and dioxins on cognitive abilities in Dutch children at 42 months of age. J. Pediatr. 134, 33-41.

Perdew, G. H., 1988. Association of the Ah receptor with the 90-kDa heat shock protein. J. Biol. Chem. 263, 13802-5.

Petersen, S. L., Curran, M. A., Marconi, S. A., Carpenter, C. D., Lubbers, L. S., McAbee, M. D., 2000. Distribution of mRNAs encoding the arylhydrocarbon receptor, arylhydrocarbon receptor nuclear translocator, and arylhydrocarbon receptor nuclear translocator-2 in the rat brain and brainstem. J. Comp. Neurol. 427, 428-39.

Peterson, R. E., Theobald, H. M., Kimmel, G. L., 1993. Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit. Rev. Toxicol. 23, 283-335.

Powell-Coffman, J. A., Bradfield, C. A., Wood, W. B. (1998). *Caenorhabditis elegans* orthologs of the aryl hydrocarbon receptor and its heterodimerization partner the aryl hydrocarbon receptor nuclear translocator. Proc. Natl. Acad. Sci. U S A 95, 2844-9.

Probst, M. R., Reisz-Porszasz, S., Agbunag, R. V., Ong, M. S., Hankinson, O. 1993. Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. Mol. Pharmacol. 44, 511-8.

Reyes, H., Reisz-Porszasz, S., Hankinson, O., 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 256, 1193-5.

Rohrig, S., Rockelein, I., Donhauser, R., Baumeister, R., 2000. Protein interaction surface of the POU transcription factor UNC-86 selectively used in touch neurons. EMBO J 19, 3694-703.

Ruvkun, G., 1997. Patterning of the nervous system. In *C. elegans* II. (ed. D.L. Riddle, T. Blumenthal, B.J. Meyer, and J.R. Priess) pp 543 – 82. Cold Spring Harbor: Cold Spring Harbor Press.

Sarafi-Reinach, T.R., Melkman, T., Hobert, O., Sengupta, P., 2001. The *lin-11* LIM homeobox gene specifies olfactory and chemosensory neuron fates in *C. elegans.* Development 128, 3269-81.

Schantz, S. L., Bowman, R. E., 1989. Learning in monkeys exposed perinatally to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Neurotoxicol. Teratol. 11, 13-9.

Schmidt, J. V., Bradfield, C. A., 1996. Ah receptor signaling pathways. Annu. Rev. Cell. Dev. Biol. 12, 55-89.

Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., Bradfield, C. A., 1996. Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. Proc. Natl. Acad. Sci. *U S A* 93, 6731-6.

Shimizu, Y., Nakatsuru, Y., Ichinose, M., Takahashi, Y., Kume, H., Mimura, J., Fujii-Kuriyama, Y., Ishikawa, T., 2000. Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc. Natl. Acad. Sci. U S A 97, 779-82.

Shirasaki, R., Pfaff, S.L. 2002 Transcriptional codes and the control of neuronal identity. Annual Rev. Neurosci. 25, 251-81.

Song, J., Clagett-Dame, M., Peterson, R. E., Hahn, M. E., Westler, W. M., Sicinski, R. R., DeLuca, H. F., 2002. A ligand for the aryl hydrocarbon receptor isolated from lung. Proc. Natl. Acad. Sci. USA 99, 14694-9.

Sulston, J. E., Horvitz, H. R., 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev. Biol. 56, 110-56.

Tam, T., Mathews, E., Snutch, T. P., Schafer, W. R., 2000. Voltage-gated calcium channels direct neuronal migration in *Caenorhabditis elegans*. Dev. Biol. 226, 104-17.

Vorderstrasse, B. A., Steppan, L. B., Silverstone, A. E., Kerkvliet, N. I., 2001. Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens

and are resistant to TCDD-induced immune suppression. Toxicol. Appl. Pharmacol. 171, 157-64.

Ward, M.P., Mosher J.T., Crews, S.T., 1998. Regulation of bHLH-PAS protein subcellular localization during Drosophila embryogenesis. Development 125, 1599-608.

Way, J. C., Chalfie, M., 1988. *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. Cell 54, 5-16.

Weinshenker, D., Wei, A., Salkoff, L., Thomas, J. H. 1999. Block of an ether-a-go-go-like K(+) channel by imipramine rescues egl-2 excitation defects in *Caenorhabditis elegans*. J Neuroscience 19, 9831-40.

White, J. G., Southgate, J. N., Thomson, J. N., Brenner, S., 1986. The structure of the nervous system of *Caenorhabditis elegans*. Philos. Trans. R. Soc. Lond. B Biol. Sci. 314, 1 - 340.

Whitlock, J. P., Jr., 1999. Induction of cytochrome P4501A1. Annu. Rev. Pharmacol. Toxicol. 39, 103-25.

Wu, J., Duggan, A., Chalfie, M., 2001. Inhibition of touch cell fate by *egl-44* and *egl-46* in *C. elegans*. Genes Dev. 15, 789-802.

Xue, D., Tu, Y., Chalfie, M., 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. Science 261, 1324-8.

Yu, S., Avery, L., Baude, E., Garbers, D. L., 1997. Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. Proc. Natl. Acad. Sci. U S A 94, 3384-7.

Yu, T. W., Hao, J. C., Lim, W., Tessier-Lavigne, M., Bargmann, C. I., 2002. Shared receptors in axon guidance: SAX-3/Robo signals via UNC-34/Enabled and a Netrin-independent UNC-40/DCC function. Nat. Neurosci. 5, 1147-54.

Zallen, J. A., Yi, B. A., Bargmann, C. I., 1998. The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in *C. elegans*. Cell 92, 217-27.

Zhang, Y., Ma, C., Delohery, T., Nasipak, B., Foat, B. C., Bounoutas, A., Bussemaker, H. J., Kim, S. K., Chalfie, M., 2002. Identification of genes expressed in *C. elegans* touch receptor neurons. Nature 418, 331-5.

Table 1. *ahr-1*-deficient animals fail to express *gcy-32*:GFP in AQR and PQR

Cell	Genetic Background	Expression level of *gcy-32*:GFP			
		% Strong	% Dim	% N.D.	n
AQR	WT	67	20	13	107
	ahr-1 (ia03)	0	2	98	104
PQR	WT	80	5	15	107
	ahr-1 (ia03)	1	4	96	113

N.D. not detectable; n = number scored. Strong: the cell body and axon fluoresced brightly; Dim: GFP fluorescence is detectable in the cell bodies, but expression in the axon is faint or not detectable; N.D. GFP is not detectable.

Table 2. ***ahr-1*** **and** ***unc-86*** **activate** ***gcy-32*****:GFP expression in the URX neurons, and** ***ahr-1*** **expression is regulated by** ***unc-86.***

GFP reporter	Genetic Background	GFP expression in URX cells			
		% Strong	% Dim	% N.D.	n
gcy-32:GFP	WT	92	2	6	126
	ahr-1 (ia03)	0	34	66	111
	unc-86 (n306)	10	23	67	132
	unc-86 (n846)	5	36	59	132
ahr-1: GFP	WT	96	4	0	102
	ahr-1 (ia03)	82	18	0	124
	unc-86 (n306)	24	59	17	112
	unc-86 (n846)	35	55	10	104

N.D. not detectable; n = number scored. Strong: the cell body and axon fluoresced brightly; Dim: GFP fluorescence is detectable in the cell bodies, but expression in the axon is faint or not detectable; N.D. GFP is not detectable.

FIGURE LEGENDS

Figure 1: *ahr-1:gfp* fusion genes are expressed in a subset of neurons during larval development. **A.** Diagram of the two *ahr-1:gfp* reporters used in this study. Exons are drawn as boxes. The PAS domain is encoded by exons 4 – 9, and the GFP sequences are inserted in exon 11 of pJ360. **B – E.** Cells labeled by expression of the pHT102 transgene. In all images, anterior is left, and ventral is down. **B.** ALNR and ALNL are visible in the hermaphrodite tail. **C.** AVM is located anterior to the vulva, in the ventral half of the animal. AVM projects a single axon that crosses the PLNR axon before reaching the ventral nerve cord and turning towards the anterior nerve ring. **D.** The SDQR cell body is located on the nerve associated with ALMR and ALNR. The SDQR axon extends to the dorsal sub-lateral nerve and then turns anteriorly. AVM is faintly visible in another focal plane. **e.** In this merged image of multiple medial confocal sections of the head, the URX neurons are visible

Figure 2: *ahr-1* function is required for appropriate anterior-posterior positioning of AVM and SDQR. **A.** Diagram depicting the migration of QR and its descendents in a wild-type animal. SDQR and AVM are sister cells, born from QR.pa. The V cells divide during these migrations to give rise to anterior and posterior daughters, labeled V.a and V.p in **C** and **D.** **B.** The *ahr-1 (ia03)* mutation is a 1517 bp deletion that removes exons 4 – 7, resulting in a frameshift and a premature stop. The deletion breakpoints are 205 bp 5' to exon 4 and 30 bp 5' to exon 8, and it does not remove coding sequences for C41G7.6, a small gene in the third intron. **C, D.** The positions of the AVM (**C**) and SDQR (**D**) cells were scored relative to the V cells in wild-type and *ahr-1 (ia03)* worms (n = 51 – 62).

Figure 3: Abnormal positioning and differentiation of the PLM neurons in *ahr-1 (ia03)* animals. Nomarski images of adult hermaphrodite tails are shown in **A, C, E.** An

arrowhead indicates the position of the anus. In each case, anterior is at left, and ventral is down. **B, D, F.** The *mec-18*:GFP touch receptor marker labels the 2 PLM cells in the tail. Only the PLML cell is visible in the focal planes shown. **A, B.** In wild-type *C. elegans*, the PLM cells are located in the posterior part of the lumbar ganglia. **C, D.** In 35% of the *ahr-1 (ia03)* animals, at least one of the PLM cells is displaced to a position as anterior as the anus (n = 120). **E, F.** In rare cases (3%), an additional *mec-18*:GFP expressing cell is present in the tail of *ahr-1* mutants.

Figure 4: SDQR and AVM neurons have cell and axonal pathfinding defects in *ahr-1 (ia03)* animals. **A, C.** The positions of SDQR and AVM in wild type *C. elegans* are depicted. **B, D.** An *ahr-1 (ia03)* animal is shown. Anterior is at left, and ventral is down. **C, D** are merged medial confocal images in which the neurons are labeled with a panneural GFP marker. **E.** The dorsal-ventral position of SDQR was scored in wild-type and mutant animals (n = 120-258). Shown here are data for wild-type, *ahr-1 (ia03),* and *aha-1 (ia01)* mutants. Also shown are the data for *ahr-1 (ia03)* animals carrying the pJ360 *ahr-1*:GFP transgene (shown in Fig 1A) and for *aha-1 (ia01)* animals rescued to viability by the pHJ32 transgene, which directs expression of AHA-1 in non-neuronal pharyngeal cells.

Figure 5: Genetic analysis of SDQR dorsal migration. **A.** The dorsal-ventral position of the SDQR cell was scored in wild-type animals and in animals carrying null mutations in *ahr-1, unc-5, unc-6, unc-40, sax-3*, or *unc-129*. *ahr-1* double mutants were also analyzed (n = 80 - 258). **B, C.** Axon projections from SDQR cells dorsal (**B.**) or ventral (**C.**) to the ALMR-associated nerve were scored. The graphs illustrate the mean and the standard error of proportion.

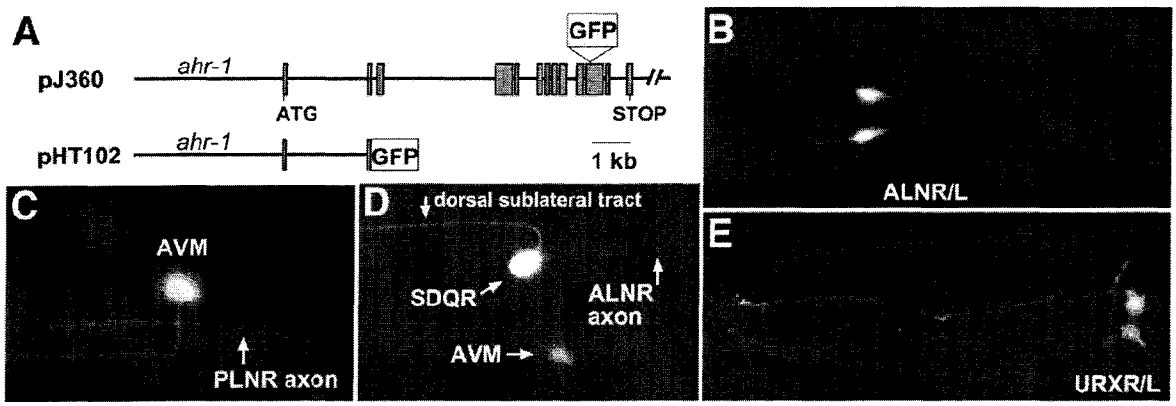
A
pJ360
ahr-1
ATG
GFP
STOP
pHT102
ahr-1
GFP
1 kb
B
ALNR/L
C
AVM
PLNR axon
D
dorsal sublateral tract
SDQR
ALNR axon
AVM
E
URXR/L

Figure 1

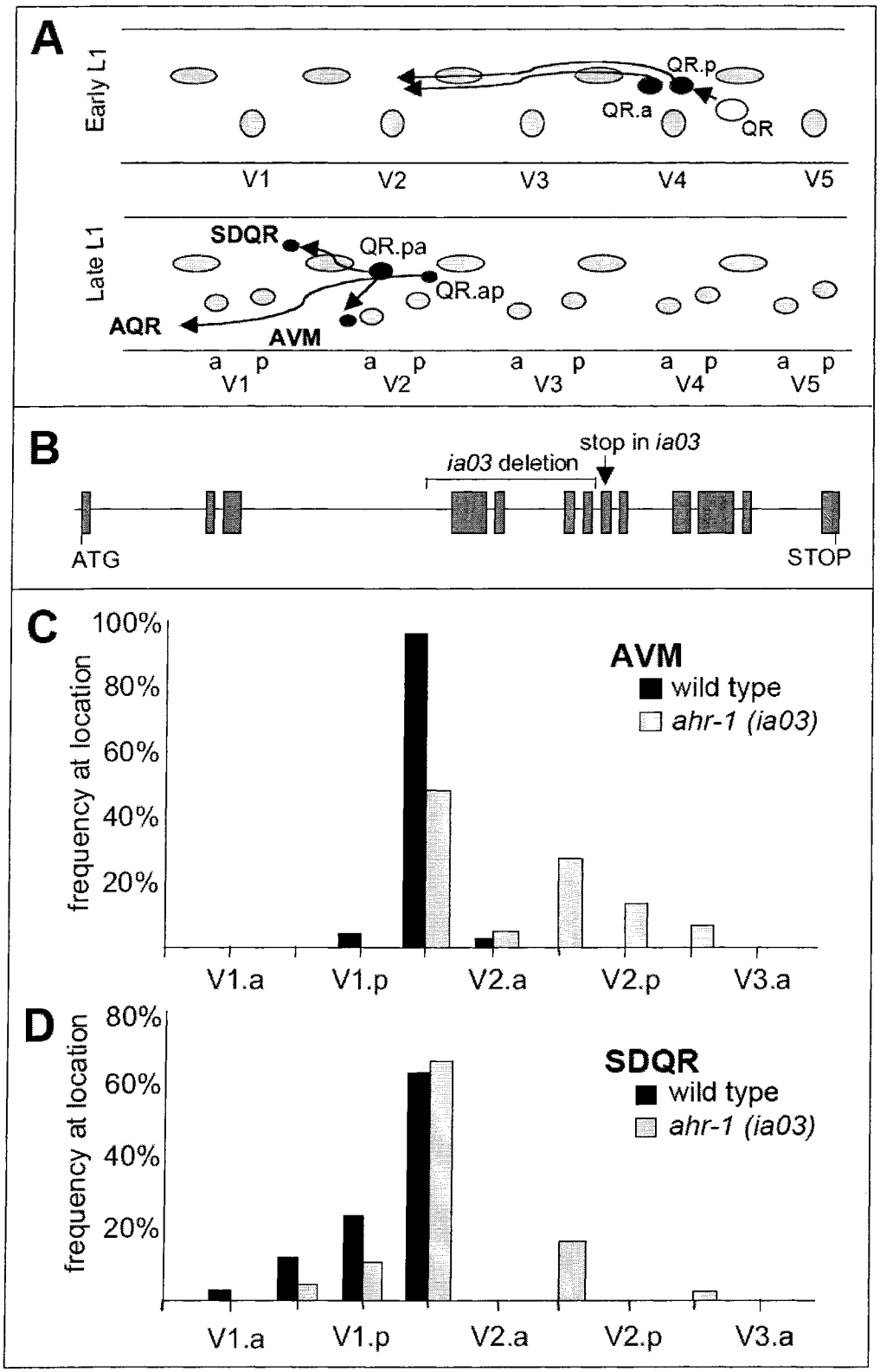
A
Early L1
QR.p
QR.a
QR
V1
V2
V3
V4
V5
Late L1
SDQR
QR.pa
QR.ap
AQR
AVM
a p
V1
V2
V3
V4
V5
B
stop in ia03
ia03 deletion
ATG
STOP
C
100%
80%
60%
40%
20%
frequency at location
AVM
wild type
ahr-1 (ia03)
V1.a
V1.p
V2.a
V2.p
V3.a
D
80%
60%
40%
20%
frequency at location
SDQR
wild type
ahr-1 (ia03)
V1.a
V1.p
V2.a
V2.p
V3.a

Figure 2

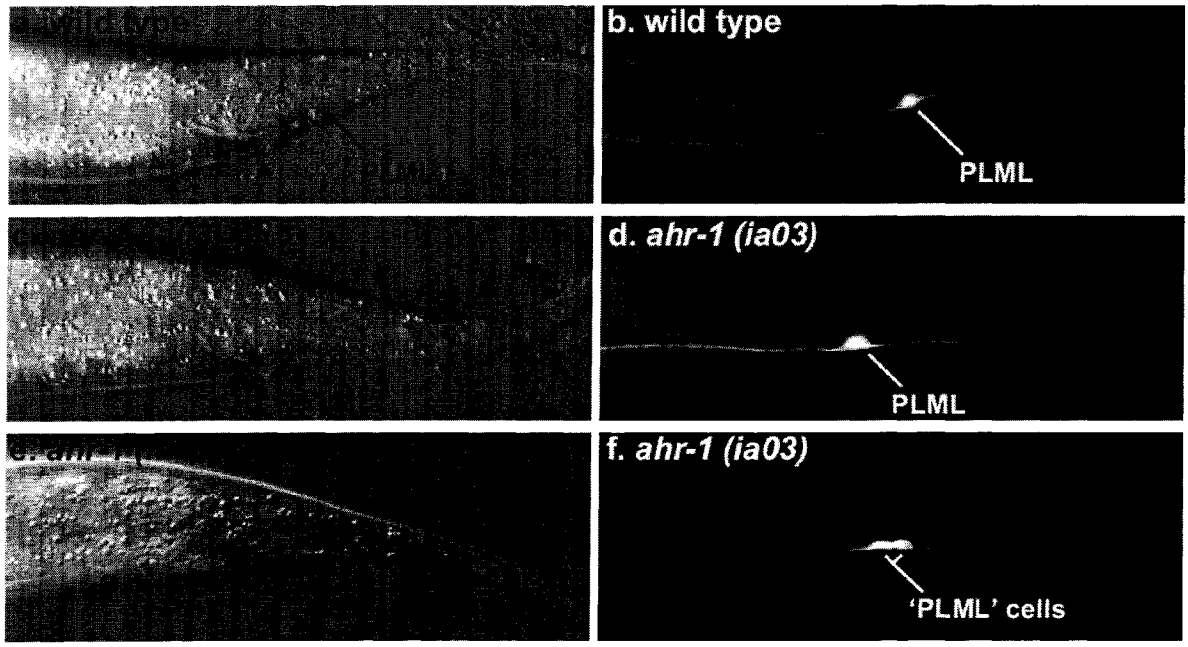

Figure3

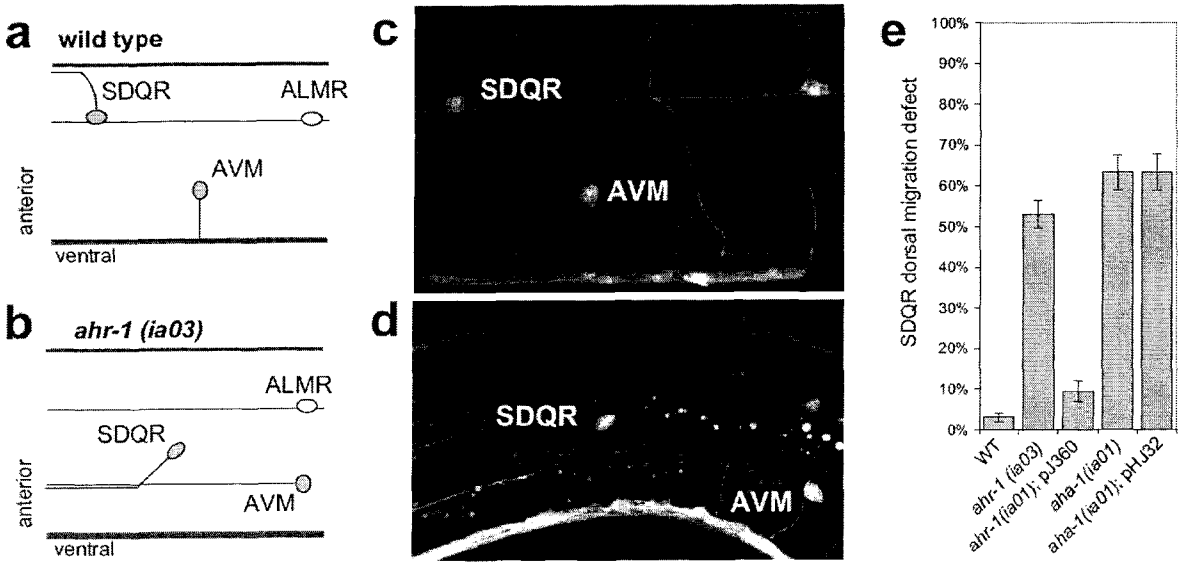
a
wild type
SDQR
ALMR
AVM
anterior
ventral
b
ahr-1 (ia03)
ALMR
SDQR
AVM
anterior
ventral
c
SDQR
AVM
d
SDQR
AVM
e
SDQR dorsal migration defect
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
ahr-1 (ia03)
ahr-1(ia01); pJ360
aha-1(ia01)
aha-1(ia01); pHJ32

Figure 4

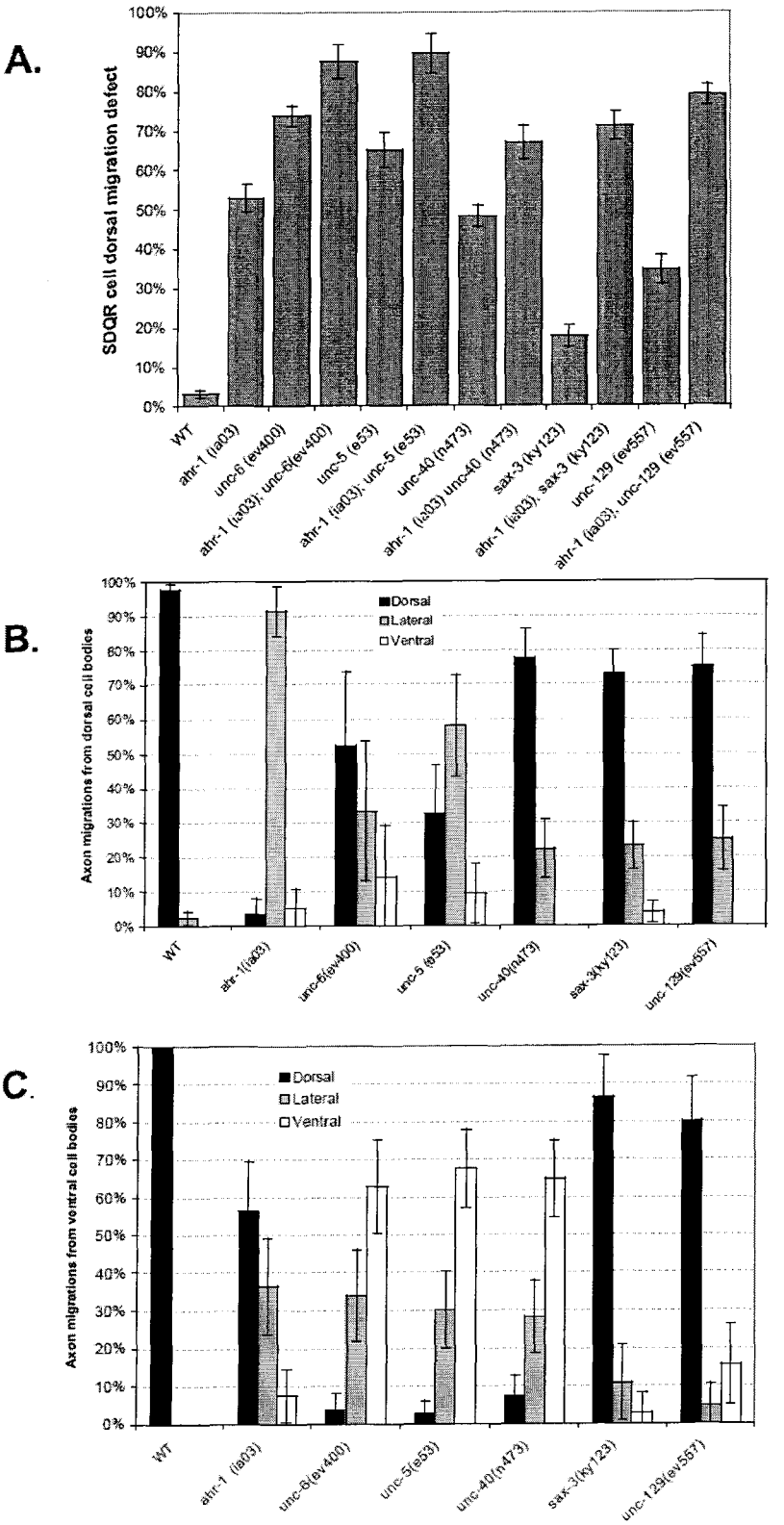
A.
SDQR cell dorsal migration defect
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
ahr-1 (ia03)
unc-6 (ev400)
ahr-1 (ia03); unc-6(ev400)
unc-5 (e53)
ahr-1 (ia03); unc-5 (e53)
unc-40 (n473)
ahr-1 (ia03) unc-40 (n473)
sax-3 (ky123)
ahr-1 (ia03); sax-3 (ky123)
unc-129 (ev557)
ahr-1 (ia03); unc-129 (ev557)
B.
Axon migrations from dorsal cell bodies
Dorsal
Lateral
Ventral
WT
ahr-1(ia03)
unc-6(ev400)
unc-5 (e53)
unc-40(n473)
sax-3(ky123)
unc-129(ev557)
C.
Axon migrations from ventral cell bodies
Dorsal
Lateral
Ventral
WT
ahr-1 (ia03)
unc-6(ev400)
unc-5(e53)
unc-40(n473)
sax-3(ky123)
unc-129(ev557)

Figure 5

CHAPTER 3

The *C. elegans* aryl hydrocarbon receptor, AHR-1, controls expression of soluble guanylate cyclases in the URX neurons and regulates aggregation behavior

Submitted

Hongtao Qin[1], Zhiwei Zhai, and Jo Anne Powell-Coffman[2]

Abstract

The aryl hydrocarbon receptor ortholog in *C. elegans*, *ahr-1*, regulates important neuronal development events, including neuronal migration, axon pathfinding and cell fate specification. Here, we report that *ahr-1* functions acutely to regulate a specific behavior: the aggregation of *C. elegans* on lawns of bacterial food. This behavior is modulated by nutritional cues and ambient oxygen levels, and aggregation is inhibited by the NPR-1 G protein-coupled neuropeptide receptor gene. We report that loss-of-function mutations in *ahr-1* or its transcription partner *aha-1* (ARNT) suppress aggregation behavior in *npr-1*-deficient animals. Expression of *ahr-1* in only 4 neurons, including URXR and URXL, restores aggregation behavior to *ahr-1* mutant animals. Mutants defective in *ahr-1* or *aha-1* express the *gcy-32, gcy-34,* and *gcy-35* soluble guanylate cyclase (sGC) reporter genes at markedly reduced levels. These genes have been shown to have key roles in aggregation or hyperoxia avoidance. We report that the requirement for *ahr-1* in social feeding behavior can be temporally uncoupled from early development of the URX neurons,

---

[1] Primary researcher and author

[2] Author for correspondence

Acknowledgement: This work was supported by the National Science Foundation.

using a heat shock inducible promoter. Collectively, these data support a model in which the AHR-1:AHA-1 transcription complex regulates the expression of sGCs and other unidentified genes that act acutely in the URX neurons to promote aggregation behavior.

Introduction

Simple behaviors are governed by defined neuronal circuits. The activities of key neurons within each circuit are influenced by genomic information, developmental history, and environmental cues. The nematode *C. elegans* has proven to be an excellent genetic system for discovery of genes and genetic networks that integrate internal and external cues to neuronal function (Hobert, 2003; de Bono and Maricq, 2005).

C. elegans "social feeding behavior" is of particular interest because it is regulated by multiple environmental cues, including nutrition and oxygen availability. Some wild type strains of *C. elegans* form groups on the border of their bacterial food source, while other strains, including Bristol N2, are solitary feeders (de Bono and Bargmann, 1998; Rogers et al., 2003). This behavioral variation correlates with allelic differences in the *npr-1* G protein-coupled neuropeptide receptor gene (de Bono and Bargmann, 1998). Strains that aggregate on food contain the NPR-1 215F isoform, which has lower activity than the NPR-1 215V isoform that is present in strains that do not aggregate (de Bono and Bargmann, 1998). Moreover, a strong loss-of-function mutation in *npr-1* is sufficient to cause Bristol N2 to exhibit "social feeding behavior" (de Bono and Bargmann, 1998). Solitary feeding behavior can be restored to *npr-1* mutants by expressing wild-type *npr-1* (the 215V isoform) in the four neurons that contact the pseudocoelomic space: URXR, URXL, AQR, and PQR. Genetic inactivation of these four neurons abolishes aggregation behavior (Coates and de Bono, 2002). In current models, activation of NPR-1 suppresses the depolarization of the neurons that contact the pseudocoelom (URXR, URXL, AQR, and PQR), thereby inhibiting aggregation on bacteria (Coates and de Bono, 2002).

Social feeding behavior is promoted by high levels of ambient oxygen. *npr-1*-deficient animals form groups on bacterial borders if incubated in room air, but aggregation behavior can be suppressed by lowering environmental oxygen levels (Gray et al., 2004). [need something here] The GCY-35 / GCY-36 soluble guanylate cylcase

complex appears to function as the oxygen sensor within the URX neurons that control social feeding behavior. GCY-35 has been shown to bind oxygen (Gray et al., 2004), and GCY-36 is thought to function as a heterodimer with GCY-35 (Cheung et al., 2004). Mutations in the *gcy-35* or *gcy-36* soluble guanylate cyclase (sGC) genes prevent aerotaxis to lower oxygen concentrations, and they also suppress "social feeding" in animals that lack *npr-1* function (Cheung et al., 2004; Gray et al., 2004; Cheung et al., 2005). Studies by our research group (described below) and by others (Cheung et al., 2004; Gray et al., 2004) indicate that *gcy-35* and *gcy-36* are expressed in a subset of neurons that includes the URX class of neurons. Thus, high levels of ambient oxygen may regulate production of cGMP by the GCY-35 complex in the URX neurons and promote aerotaxis to less hyperoxic microenvironments.

Interestingly, a strong loss-of-function mutation in *ahr-1* suppresses social feeding behavior (Qin and Powell-Coffman, 2004). *ahr-1* is orthologous to the mammalian aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that mediates the toxic effects of dioxins and certain other environmental pollutants (Powell-Coffman et al., 1998; Gu et al., 2000). In addition to its xenobiotic sensor function, AhR also has important developmental functions (Crews, 2003). Inappropriate activation of AhR by dioxin or other manmade chemicals can cause cancer, cleft palate, neurological defects, and a range of other pathologies (Pohjanvirta and Tuomisto, 1994; Denison and Heath-Pagliuso, 1998; Birnbaum and Tuomisto, 2000; Mandal, 2005). Mice lacking AhR function exhibit developmental abnormalities (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997), and mice expressing a constitutively active form of AhR are predisposed to stomach cancer (Andersson et al., 2002). These studies suggest that molecules that regulate AhR function could have important developmental or physiological roles.

The *C. elegans* orthologs of AhR and ARNT are encoded by the *ahr-1* and *aha-1* genes (Powell-Coffman et al., 1998). *C. elegans* AHR-1 is expressed in a subset of neurons, and

worms lacking *ahr-1* function have a range of neurological defects (Huang et al., 2004; Qin and Powell-Coffman, 2004). These studies, when considered with the neurotoxic effects of certain AhR-activating ligands, suggest that the aryl hydrocarbon receptor may have ancient roles in neuronal development and function.

Previous studies of the *ahr-1* expression pattern and mutant phenotype provided some insight to the mechanism(s) by which *C. elegans* AHR-1 might promote social feeding behavior. AHR-1 is expressed in a subset of neurons, including the URX cells, which have a central role in the neural circuit that governs aggregation behavior. In *ahr-1* mutants the URX neurons develop with normal morphology and express a panneural marker, but two markers of URX differentiation (*gcy-32*:GFP and *npr-1*:GFP) are expressed at markedly reduced levels compared to wild-type animals (Qin and Powell-Coffman, 2004). Coates and de Bono (2002) have shown that genetic inactivation of the URX class of neurons suppresses *npr-1*-mediated social feeding behavior. Therefore, the behavioral phenotype of *ahr-1* mutants could be explained by either of two nonexclusive models. First, mutation of *ahr-1* impaired the ability of the URX cells to develop into functional neurons. In this model, *ahr-1* did not have a direct role in the regulation of aggregation behavior. Alternatively, AHR-1 might promote the expression of genes integral to the cellular networks in URX that control social feeding behavior.

In this study, we interrogate *ahr-1* function in the URX neurons. We show that post-embryonic expression of *ahr-1* is sufficient to enable social feeding behavior. We demonstrate that *ahr-1* promotes the expression of soluble guanylate cyclase (sGC) genes that are key regulators of aggregation behavior. This provides a conceptual framework for understanding how molecules that modulate aryl hydrocarbon receptor function can influence a simple behavior.

Materials and Methods

C. elegans culture and behavioral assays

C. elegans were cultured using standard methods (Brenner, 1974). The wild-type parent for the strains used in this study was *C. elegans var. Bristol* strain N2. The following mutant alleles were used in this study: linkage group I (LGI): *aha-1(ia01), ahr-1(ia03), ahr-1(ju145), dpy-5(e61), unc-119(ed3);* LGIV: *daf-21(673), daf-21(nr2081);* LGX: *npr-1(ky13), npr-1(ad609), npr-1(n1353).* The transgenic strains carried integrated transgenes were: ZG628 *[aha-1(ia01), Pcky-1:aha-1(iaIs18), unc-119(+)]*; ZG611 *[Pgcy-32:gfp(iaIs19), unc-119(+)]*; ZG605 *[Pgcy-34:gfp(iaIs20), unc-119(+)]*; ZG601 *[Pgcy-35:gfp(iaIs21), unc-119(+)]*; ZG629 *[Pgcy-36:gfp(iaIs22), unc-119(+)]*; ZG630 *[mPgcy-35:gfp(iaIs23), unc-119(+)]*; ZG631 *[mPgcy-36:gfp(iaIs24), unc-119(+)]*; ZG610 *[Pgcy-37:gfp(iaIs25), unc-119(+)]*.

Social feeding assays were performed as previously described (de Bono et al., 2002; Gray et al., 2004). Forty worms were scored in each experiment. Each genotype was assayed in at least six independent experiments.

The fluorescence of GFP reporters was examined on a Nikon E800 compound fluorescence microscope equipped with DIC optics. Worms were mounted on 4% agarose pads in M9 buffer containing 5mM sodium azide.

Molecular Biology

The pHT108 plasmid includes *ahr-1* genomic and cDNA sequences. To construct pHT108, a 5377 bp HindIII-BamHI genomic fragment, including over 3 kb of sequence 5' to translational start codon, exon 1, intron 1, and part of exon 2, was fused to a cDNA fragment containing the remaining coding sequences. The cDNA insert was a HindIII-BamHI fragment from pJ345 (Powell-Coffman et al., 1998). To add *ahr-1* 3'

genomic sequences, this construct was cut with BstXI (in exon 11) and BglII, and then ligated with the 1260 bp BstXI-BglII genomic fragment from the pJ301 plasmid (Qin and Powell-Coffman, 2004).

To construct the pHT111 *hsp16.2::ahr-1* plasmid, the 516 bp HindIII-NheI *hsp16.2* promoter fragment from plasmid pPD49.78 (a gift from Andy Fire) was cloned into the HindIII-NheI sites in the polylinker of pHT110. The pHT110 plasmid is a promoterless construct that includes full length *ahr-1* cDNA sequence and 3' genomic sequences. The pHT110 construct was engineered as follows: 6824 bp HindIII-BstXI pHT108 fragment was ligated with the ~1500bp HindIII-BstXI pJ345 fragmant. This construct was then cut by HindIII and ligated to an oligonucleotide polylinker: 5'AGC TTG TCG ACC CAT GGC CCG GGG GTA CCA AGC TAG CGA ATT CC 3' and 5'AGC TGG AAT TCG CTA GCT TGG TAC CCC CGG GCC ATG GGT CGA CA 3'.

To construct the pHT112 *flp-8::ahr-1* plasmid, a 3203 bp genomic fragment, including 3187 bp sequences 5' to *flp-8* start codon and 16 bp sequences in the first exon, was ligated to the XhoI-NheI sites of pHT110. The primers used to amplify the *flp-8* regulatory fragment were: 5' CAA CTC GAG CGT GAT GGA GTT CGC AGC AAT G 3' and 5' AAG CTA GCG GAC TCC GGA GAG CAC TTT CTA C 3'.

The pHT103 *Pgcy-34::gfp* plasmid was generated by ligating the 747 bp of the genomic sequence 5' to the *gcy-34* start codon to the BbuI-BamHI sites of pPD95.75 (a gift from Andy Fire). To construct the pHT106 *Pgcy-32::gfp* plasmid, a genomic fragment containing 1096 bp 5' to the *gcy-32* start codon was ligated to the PstI-BamHI sites of pPD95.75. The pHT115 *Pgcy-35::gfp* plasmid, includes 1250 bp 5' to the *gcy-35* start codon. This genomic fragment was inserted into the PstI-BamHI sites of pPD95.75. The pHT116 *Pgcy-36::gfp* plasmid includes 1172 bp of *gcy-36* of genomic sequence 5' to the start codon inserted into the PstI-BamHI sites of pPD95.75. To construct pZW2 *Pgcy-37::gfp* plasmid, a genomic fragment 1122 bp upstream to *gcy-37* start codon was ligated to the BamHI-KpnI sites of pPD95.75.

To construct the pHT117 *mPgcy-35::gfp* plasmid, a C to T point mutation was introduced into a site 111 bp upstream to the start codon of *gcy-35* in pHT115 using a PCR based approach (Ausubel, 1994). The pHT118 *mPgcy-36::gfp* plasmid was generated by introducing a G to A point mutation to the pHT116 plasmid, 263 bp upstream to the putative start codon of *gcy-36.*

Gel shift assays

Gel shift assays were performed as previously described (Powell-Coffman et al., 1998). Proteins were expressed in rabbit reticulocyle lysates (TNT coupled reticulocyte lysate system, Promega). The Pgcy-35XRE probe was derived from the *gcy-35* promoter: 5' GAT CAT CTG ACA CGC AAT GGC TCC CCG CCA CCC C annealed to 5' AGT CGG GGT GGC GGG GAG CCA TTG CGT GTC AGA T. The mPgcy-35XRE competitor was 5' GAT CAT CTG ACA CGT AAT GGC TCC CCG CCA CCC C annealed to 5' AGT CGG GGT GGC GGG GAG CCA TTA CGT GTC AGA T. The Pgcy-36XRE probe was derived from the *gcy-36* promoter: 5' GAC TAC AGC ATT GCG TGT CTA TTA ATT ATT CAA annealed to 5' TGC ATT GAA TAA TTA ATA GAC ACG CAA TGC TGT. The mPgcy-36XRE competitor was 5' GAC TAC AGC ATT ACG TGT CTA TTA ATT ATT CAA annealed to 5' TGC ATT GAA TAA TTA ATA GAC ACG TAA TGC TGT.

Transgenic animals

To integrate constructs into the *C. elegans* genome, we employed microparticle bombardment (Praitis et al., 2001; Berezikov et al., 2004). We used a BioRad Biolistic PDS-1000/He system with 28 inches of Hg vacuum, 650 p.s.i. rupture disks, and 0.6μm gold particles. For each bombardment, 2μg pPDMM016 (*unc-119* co-transformation marker) (Praitis et al., 2001) plasmid DNA and 1μg target plasmid DNA were bombarded into *unc-119(ed3)* L4 and young adult hermaphrodites.

To generate extrachromosomal arrays of transgenes, DNA was microinjected into young adult *dpy-5(e61)* hermaphrodite germlines, following standard procedures (Epstein et al., 1995). The *dpy-5* plasmid pCes-361 (a gift from Dr. Ann Rose, University of British Columbia) was used as the coinjection marker.

Results

To rigorously examine the role of *ahr-1* in *npr-1*-mediated aggregation behavior, we characterized the phenotypes of multiple single and double mutant combinations. Previously, we had shown that *ahr-1 (ia03)* suppressed aggregation in *npr-1 (n1353)* animals (Qin and Powell-Coffman, 2004). The NPR-1 receptor is predicted to be a G protein-coupled receptor with 7 transmembrane domains. The *npr-1 (n1353)* allele is a missense mutation in transmembrane domain 3 (de Bono and Bargmann, 1998). Phenotypic analyses have confirmed that the *n1353* mutant allele is not null for *npr-1* function (de Bono and Bargmann, 1998; Wang and Wadsworth, 2002). We examined two other mutations that have been shown to cause stronger aggregation phenotypes [*npr-1 (ky13) and npr-1 (ad609)*]. The *ky13* mutation introduces a premature stop codon after the first transmembrane domain. The *ad609* allele includes two missense mutations that are predicted to cause a threonine to isoleucine substitution in transmembrane domain 2 and a threonine to alanine substitution in transmembrane domain 4 (de Bono and Bargmann, 1998). As shown in Figure 1A, the *ahr-1 (ia03)* deletion allele suppressed aggregation phenotypes caused by both of these strong loss-of-function *npr-1* alleles. Seventy-nine percent of *npr-1 (ky13)* single mutant animals and 71% of *npr-1 (ad609)* animals fed in groups, but double mutants carrying the *ahr-1 (ia03)* mutation showed marked decreases in this behavior (15% for *ky13* and 11% for *ad609*).

We anticipated that a different loss-of-function allele of *ahr-1* should also suppress *npr-1* mediated aggregation behavior. To test this, we analyzed feeding behaviors of *ahr-1 (ju145)* mutants. The *ahr-1 (ia03)* deletion removes most of the PASA domain and introduces a premature stop codon (Qin and Powell-Coffman, 2004). The *ahr-1 (ju145)* mutation introduces a premature stop codon in the PAS domain of AHR-1 (Huang et al., 2004). Thus, molecular analyses suggest that both *ia03* and *ju145* abolish *ahr-1* function. Characterization of SDQR cell migration and AVM axon pathfinding defects in these two

mutant backgrounds further suggests that *ju145* and *ia03* are both strong loss-of-function alleles (data not shown). As expected, the *ahr-1 (ju145)* mutation suppresses aggregation phenotypes in *npr-1 (ky13)* animals (Figure 1A).

Mutations in *npr-1* cause both "grouping" and "bordering" behaviors on bacterial lawns. "Grouping", the behavior quantitated in Fig. 1A, is defined as the percentage of animals feeding in groups of three or more. *npr-1*-deficient animals have also been shown to feed preferentially at the border of the bacterial lawn, where the oxygen concentration is lower (Gray et al., 2004). Consistent with prior studies (de Bono and Bargmann, 1998; de Bono et al., 2002), we found that 95% *npr-1(ky13)* or *npr-1(ad609)* animals exhibited "bordering" behavior. Strong loss-of-function mutations in *ahr-1 (ia03* or *ju145)* significantly suppressed this behavior (to 65% and 66% respectively; Fig 1B). Both grouping and bordering behavior can be restored to *ahr-1; npr-1* double mutants by the introduction of the pHT108 *ahr-1* minigene, in which *ahr-1* genomic sequences are fused to cDNA sequences (*Pahr-1:ahr-1*; Fig. 1C and 1D). Collectively, these studies provide strong evidence that *npr-1*-mediated grouping and bordering behaviors require *ahr-1* function.

AHR-1 has been shown to form a heterodimeric DNA binding complex with AHA-1, the *C. elegans* ortholog of mammalian ARNT (Powell-Coffman et al., 1998), and our working hypothesis was that AHR-1 functioned within this complex to modulate feeding behavior. To test this hypothesis, we analyzed aggregation behavior in *aha-1* loss-of-function mutations. Like its mammalian cognate, AHA-1 forms complexes with multiple bHLH-PAS proteins, and AHA-1 is expressed in most, if not all, cells (Powell-Coffman et al., 1998; Jiang et al., 2001). *aha-1 (ia01)* is a strong loss-of-function allele, and *C. elegans* that are homozygous for the *ia01* mutation arrest development as young larvae. Interestingly, mosaic animals that express *aha-1* in non-neuronal pharyngeal cells from the *cky-1* promoter can develop to adults (Jiang, Wu, Qin, and Powell-Coffman, unpublished observations). As shown in Fig. 1A, aggregation behavior is diminished in

npr-1 (ky13) or *npr-1 (ad609)* animals that are *aha-1*-deficient. These data confirm that AHA-1, the transcriptional partner for AHR-1, has an important role in aggregation behavior.

AHR-1 functions in the URX neurons to promote aggregation

Although the neural circuits that control aggregation behaviors have not been fully elucidated, some of the neurons that regulate grouping and bordering behavior have been identified (Coates and de Bono, 2002; de Bono et al., 2002). Only four of these cells (URXR, URXL, AQR, and PQR) have been shown to express *ahr-1* (Qin and Powell-Coffman, 2004). Coates and de Bono (2002) showed that inactivation of the URXR, URXL, AQR, and PQR neurons via the expression of a constitutively active form of the EGL-2 potassium channel greatly suppressed *npr-1*-mediated aggregation. Thus, we considered the possibility that the AHR-1 transcription complex might act cell autonomously in one or more of these four neurons to promote grouping and bordering behaviors.

To determine whether expression of *ahr-1* in the URX neurons was sufficient to rescue social feeding behavior in *ahr-1; npr-1* double mutants, we created a chimeric gene, in which the *flp-8* promoter directed expression of *ahr-1* coding sequences. *flp-8*::GFP is expressed in URX neurons and only two other cells: AUA, and PVM (Coates and de Bono, 2002; Kim and Li, 2004). Importantly, expression of *flp-8*::GFP (and therefore *flp-8::ahr-1*) is not dependent upon *ahr-1* function (data not shown). Only 5% of *ahr-1 (ia03); npr-1 (ky13)* double mutants feed in groups of three or more. Introduction of the *flp-8*::*ahr-1* transgene increases aggregation behavior over 5-fold (28% of the animals feed in groups) (Fig 1C). By comparison, introduction of the *ahr-1* minigene increases grouping almost 10-fold (to ~50%). In control studies, *npr-1 (ky13)* animals carrying only the co-injection marker exhibit 64% grouping. Thus, *ahr-1* appears to function in the URX neurons to regulate social feeding, although this data does not preclude possible roles in other neurons that may potentiate this

behavior.

Neuronal development versus neuronal function

We considered two nonexclusive models for the role of AHR-1 in the URX neurons. First, AHR-1 might function during a critical developmental period to regulate URX development. This model postulates that *ahr-1 (ia03)* mutants fail to aggregate because the URX neurons did not develop properly. Alternatively, the AHR-1 transcriptional complex might promote aggregation behavior acutely. In this model, URX neurons in *ahr-1 (ia03)* animals do not have major developmental or structural defects that impair function, but failure to express AHR-1 target genes would limit neuronal response to aggregation cues.

To distinguish between these two models, we designed an experiment to uncouple the possible developmental roles of AHR-1 from later functional requirements. The URX neurons are born during embryogenesis (White et al., 1986). *npr-1*-deficient worms exhibit grouping behavior as early as the second larval stage, and this indicates that URX and the neuronal circuits that direct aggregation are functional at this early larval stage (data not shown; Fig 2). To allow temporal control of *ahr-1* expression, we fused the *hsp-16.2* promoter to *ahr-1* coding sequences. Expression from the *hsp-16.2* promoter is induced by high temperature, and expression is specific to neurons (Stringham et al., 1992). In control experiments, we determined that this transgene had little effect on the aggregation behavior of *ahr-1, npr-1* double mutants in the absence of heat shock induction (Figure 2). We allowed the animals to progress to the last larval stage (L4) and then exposed them to heat shock (30°C for 30 minutes). This resulted in a significant increase in aggregation behavior of *ahr-1(ia03); npr-1(ad609)* and *ahr-1(ia03); npr-1(ky13)* animals (from 6% to 38% and 6% to 31% respectively). Heat shock induction of *ahr-1* expression in late-stage (3-fold) embryos, L1 larvae, or L2 larvae also restored aggregation behavior to *ahr-1; npr-1* double mutant worms (data not shown). These data show that expression of AHR-1 after

the URX neurons are formed is sufficient to restore aggregation behavior to *npr-1*-defective animals.

ahr-1 regulates the expression of guanylate cyclase genes in URX neurons.

Soluble guanylate cyclases (sGCs) produce the 3', 5' -cyclic guanosine monophosphate (cGMP) second messenger. sGCs were good candidates for AHR-1 target genes that regulate URX activity and social feeding. The TAX-2 / TAX-4 cGMP-gated sensory channels are expressed in the URX neurons and promote social feeding behavior (Coburn and Bargmann, 1996; Komatsu et al., 1996; Coates and de Bono, 2002). Seven predicted sGCs had been identified in the *C. elegans* genome (Morton et al., 1999). We had previously shown that expression of *gcy-32*:GFP was regulated by AHR-1 (Qin and Powell-Coffman, 2004). To address the hypothesis that *ahr-1* regulated the expression of other sGC genes, we constructed GFP reporters in which the upstream regulatory sequences for each sGC gene was fused with the coding sequences of green fluorescent protein (GFP). Initially, we generated transgenic animals carrying extrachromosomal arrays of these reporters (Table 1). We later confirmed the expression patterns in animals in which the reporters were integrated into the genome (Table 2). Consistent with the independent studies of Cheung *et. al.* (2004) and Gray *et. al.* (2004), we found that the GFP reporters for 5 sGC genes, *gcy-32, gcy-34, gcy-35, gcy-36,* and *gcy-37,* were consistently expressed in the AQR, PQR, URXL and URXR neurons. Expression of *gcy-37*:GFP was also observed in AVM and two unidentified neurons located in the head. *gcy-35*:GFP was expressed in fifteen neurons, all of which also express *ahr-1*: AQR, PQR, URXR/L, ALNL/R, BDUL/R, SDQL/R, PLML/R, AVM and two neurons in the tail tentatively identified as PLNL/R. (*gcy-35*:GFP was only transiently expressed in PLML/R during the first larval stage.) To determine whether *ahr-1* function was required for the expression of these sGCs, we compared expression levels of the GFP reporters in wild-type and *ahr-1 (ia03)* animals. As shown in Tables 1 and 2, animals carrying the *ahr-1 (ia03)* mutation

expressed the *gcy-32*, *gcy-34*, and *gcy-35* reporters at dramatically reduced levels. Loss of *ahr-1* function had lesser effect on expression of the *gcy-36* and *gcy-37* reporters.

These data suggested that certain sGC genes were activated, directly or indirectly, by the AHR-1 transcriptional complex. To confirm that AHA-1 (the transcription factor dimerization partner for AHR-1) also promoted expression of the sGC genes, we assayed the expression of the sGC reporters in animals that were defective for *aha-1* function. The data shown in Table 2 confirmed that *aha-1* function was required for the expression of *gcy-32:GFP*, *gcy-34:GFP* and *gcy-35:GFP* reporters in URX neurons, and had a lesser role in the regulation of *gcy-36:GFP* and *gcy-37:GFP* expression.

Expression of *ahr-1*-dependent reporters in URX does not require *daf-21* / HSP90.

The HSP90 heat shock protein associates with mammalian AHR proteins, and HSP90 is thought to have an important role in AHR folding and function (Pongratz et al., 1992; Petrulis and Perdew, 2002). *C. elegans* AHR-1 has been shown to interact with rabbit HSP90 *in vitro* (Powell-Coffman et al., 1998). *daf-21* is the sole *C. elegans* ortholog of HSP90, and it has essential functions during larval development (Birnby et al., 2000). Interestingly, strong loss-of-function mutations in *daf-21* do not alter differentiation of the RMEL/R neurons, although theses cell fate decisions are dependent upon *ahr-1* function. These data indicate that in the absence of HSP90 chaperones, *C. elegans* AHR-1 is still able to fold, translocate to the nucleus, and form an active transcription complex in RME neurons (Huang et al., 2004). To further test the hypothesis that AHR-1 function in *C. elegans* does not require *daf-21*/HSP90, we analyzed the expression level of *gcy-32*:GFP, *gcy-34*:GFP and *gcy-37*:GFP in *daf-21 (nr2081)* and *daf-21 (p673)* mutants. As shown in Table 2, the *daf-21* mutations had no detectable effect on the expression of these three reporters. Thus, AHR-1 can function to up-regulate the expression of these sGC reporters in the URX neurons in the absence of *daf-21* / HSP90 function.

Multiple transcriptional targets for the AHR-1 complex in the URX neurons.

During the course of these studies, the Bargmann and de Bono groups discovered that *gcy-35* and *gcy-36* are key regulators of hyperoxia avoidance and "social feeding" (Cheung et al., 2004; Gray et al., 2004). Remarkably, GCY-35 was shown to bind oxygen, and GCY-35 likely functions in a heterodimeric complex with GCY-36. Current models suggest that the GCY-35:GCY-36 complex produces cGMP in an oxygen-dependent manner. This would activate the TAX-2 / TAX-4 channels, which mediate hyperoxia avoidance and aggregation behavior (Cheung et al., 2004; Gray et al., 2004; Cheung et al., 2005).

These data suggested that the failure of *ahr-1* mutants to express *gcy-35* and *gcy-36* at normal levels might be sufficient to inhibit *npr-1*-mediated aggregation, but it was also possible that AHR-1 had other important roles in the regulation of aggregation behavior. To distinguish between these models, we employed two experimental approaches. First, we tested the hypothesis that the AHR-1:AHA-1 complex bound directly to DNA binding sites in the *gcy-35* and *gcy-36* upstream regulatory regions. Second, we tested the hypothesis that expression of *gcy-35* and *gcy-36* from heterologous, AHR-1-independent promoters would be sufficient to restore aggregation behavior to *ahr-1; npr-1* double mutants.

We identified potential XRE's (xenobiotic regulatory elements; AHR-1:AHA-1 binding sites) in the putative 5' regulatory sequences for all five sGC genes that are expressed in URX (Figure 3). To determine whether AHR-1 and AHA-1 could interact to bind putative XREs 5' to the *gcy-35* and *gcy-36* translational start sites, we employed electrophoretic mobility shift assays. We expressed AHR-1 and AHA-1 in rabbit reticulocyte lysates (Powell-Coffman et al., 1998). As expected, oligonucleotide probes containing the XRE sequences did form slower mobility complexes when co-incubated with lysates that contained both AHR-1 and AHA-1 (data not shown). The complexes could be supershifted by co-incubation of a monoclonal antibody specific to AHA-1 (Jiang et al.,

2001). Unlabeled oligonucleotides containing a point mutation in the core AHR-1 halfsite (G --> A; Fig 3) did not compete effectively for binding to this complex (data not shown).

To determine whether the putative XREs were important for AHR-1-mediated expression of transgenes *in vivo*, we introduced mutations into the putative XREs of the *gcy-35*:GFP and *gcy-36*:GFP reporters (Figure 3). These constructs were integrated into the genome using microparticle bombardment. Interestingly, expression from the mutated reporters was still regulated by *ahr-1*, even though we had mutated the putative XREs. As shown in Table 2, expression of *mPgcy-35:GFP* (in which the putative XRE was mutated) dropped markedly when the *ahr-1 (ia03)* mutation was crossed into the strain. High levels of *mPgcy-36:GFP* expression also required *ahr-1* and *aha-1* function. Thus, mutation of the putative XREs did not abrogate regulation by the AHR-1:AHA-1 transcription factor. This indicates that the AHR-1:AHA-1 complex is able to regulate *gcy-35* and *gcy-36* expression via other promoter sequences. It is possible that there is another cryptic AHR-1:AHA-1 binding site in the *gcy-35* and *gcy-36* promoters. It is perhaps more likely that the AHR-1 complex acts indirectly to promote *gcy-35* and *gcy-36* expression.

Are other AHR-1 target genes required to promote social feeding behavior? To address this, we tested the hypothesis that expression of *gcy-35* and *gcy-36* in the URX neurons was sufficient to restore the aggregation behavior to *ahr-1, npr-1* double mutant worms. To enable expression of *gcy-35* and *gcy-36* independent of *ahr-1* function, we employed the *flp-8* promoter. Expression of *ahr-1* from the *flp-8* promoter is sufficient to cause a ~5-fold increase in the number of *ahr-1, npr-1* double mutants that feed in groups (Figure 1). To monitor *gcy-35* and *gcy-36* expression in these experiments, we incorportated synthetic operons in which the *gcy* genes were co-expressed with GFP (Cheung et al., 2004). In otherwise wild-type animals, the *flp-8* promoter directed GFP expression in the predicted pattern (URX, AUA and PVM neurons). As described above, the *ahr-1 (ia03)* mutation suppressed *npr-1*-mediated social feeding, and aggregation behavior could be largely restored by expression of *ahr-1* from the *flp-8* promoter (Figure 1). However, the

flp-8:gcy-35 and *flp-8:gcy-36* transgenes, individually or in combination, were not able to increase grouping or bordering in *ahr-1, npr-1* double mutants (Figure 4). These findings suggest that AHR-1 regulates the expression of *gcy-35*, *gcy-36*, and at least one other gene that has an essential role in aggregation behavior.

Discussion

Studies of human populations that were exposed to high levels of 2,3,7,8 tetrochlorodibenzo-p-dioxin during the Vietnam War or after industrial accidents have revealed a correlation between high levels of serum dioxin and memory deficits (Barrett et al., 2001; Pelclova et al., 2001), but the role of AhR in cognitive function has been enigmatic. Dioxin exposure causes a wide range of defects in the developing vertebrate nervous system (Birnbaum and Tuomisto, 2000; Dong et al., 2001; Hays et al., 2002). Given the complexity of the mammalian nervous system, it will be very challenging to elucidate the molecular mechanisms by which AhR influences neural function. The AhR orthologs in *C. elegans* and in *Drosophila* are required for normal neural development, and this suggests that there may be an ancestral function for AHR in neurons (Duncan et al., 1998; Huang et al., 2004; Qin and Powell-Coffman, 2004). Our present data describe an essential role for the AHR-1 transcriptional complex in a specific behavior, in which *C. elegans* form groups on the border of the bacterial food source.

The key findings of this study are: 1) expression of *ahr-1* in only 4 neurons, including URXR and URXL, is sufficient to restore aggregation behavior to *ahr-1* mutant animals; 2) social feeding behavior can be rescued in *ahr-1; npr-1* double mutants by induction of *ahr-1* expression from a heat-shock promoter after the URX neurons have formed; 3) *ahr-1* and *aha-1* promote the expression of sGC genes that have been shown to regulate hyperoxia avoidance or aggregation behavior; 4) expression of *gcy-35* and *gcy-36* is not sufficient to rescue the *ahr-1* mutant phenotype, and this indicates that the AHR-1:AHA-1 transcriptional complex is required for the appropriate expression of other, unidentified genes that enable social feeding behavior. These findings support the model shown in Figure 4.

To understand the role of AHR-1 in this behavior, it was of central importance to determine whether AHR-1 was required in URX cells. However, we could not employ the

gcy-32 promoter, since *gcy-32* is regulated by *ahr-1* function (Qin and Powell-Coffman, 2004). Instead, we constructed a chimeric gene, *flp-8::ahr-1*, that directed *ahr-1* expression in only four neurons: URXR, URXL, AUA, and PVM. Expression of *ahr-1* from the *flp-8* promoter increased grouping in *ahr-1; npr-1* double mutants by over five fold (Figure 1C). *ahr-1* reporter constructs are not detectably expressed in the AUA neuron (Qin and Powell-Coffman, 2004), and the PVM neuron is not known to have a role in aggregation behavior (Coates and de Bono, 2002; de Bono et al., 2002). Thus, we strongly favor a model in which AHR-1 is principally required in the URX neurons.

Mutants defective in *ahr-1* or *aha-1* express key regulators of hyperoxia avoidance and social feeding behavior at decreased levels. Analyses of GFP reporters indicate that *ahr-1* and *aha-1* promote the expression of *gcy-32, gcy-34, gcy-35, gcy-36, gcy-37* (Table 2) and *npr-1* (Qin and Powell-Coffman, 2004). We found possible DNA binding sites for AHR-1:AHA-1 in the regulatory sequences for each sGC examined, and this suggested that the sGC genes might be direct targets of the AHR-1 complex. However, mutation of the putative XREs in *gcy-35* and *gcy-36* did not abolish regulation of the reporters by AHR-1. These results do not preclude a role for the putative XREs in sGC expression, nor do they rule out possible cryptic AHR-1:AHA-1 binding sites. Optional binding sites have not been determined. We favor a model in which AHR-1 acts upstream of other unidentified factors to induce expression of *gcy-35* and *gcy-36* (Figure 4).

Expression of *gcy-35* and *gcy-36* was not sufficient to restore social feeding behavior to *ahr-1* mutants. This result indicates that additional genes regulated by AHR-1 have central roles in hyperoxia avoidance or social feeding. Recent data provide some insights to other functions of genes regulated by *ahr-1*. Single mutations in *gcy-32*, *gcy-34* or *gcy-37* do not exhibit aggregation defects (Cheung et al., 2004). However, *gcy-32; gcy-34* double mutant does (Cheung et al., 2005). Cheung et al (2005) demonstrated that *gcy-32* and *gcy-34* also have roles in aerotaxis behaviors. *C. elegans* that have been cultured in standard laboratory conditions prefer 5 – 12 % oxygen (Gray et al., 2004). However, if *C. elegans*

are provided with bacterial food at 1% oxygen, then the animals prefer lower oxygen concentrations (Cheung et al., 2005). In the wild, *C. elegans* live in the soil, and a preference for low oxygen levels may guide them to microenvironments with abundant bacterial or fungal food. The other genes that are regulated by the AHR-1 transcriptional complex may also have roles in sensing oxygen and nutritional cues or in behavioral responses to these environmental stimuli.

C. elegans AHR-1 and AHA-1 have been shown to bind XRE sequences with specificity, and it is clear that *C. elegans* AHR-1 and human AhR were derived from a common ancestral protein. Like other invertebrate AhR homologs that have been tested, *C. elegans* AHR-1 does not bind 2,3,7,8 tetrachlorodibenzo-p-dioxin or β-naphthoflavone, although both of these compounds bind and activate mammalian AhR (Powell-Coffman et al., 1998; Butler et al., 2001). *C. elegans* AHR-1 can also function in the absence of *daf-21* / HSP90 (Table 2; Huang et al., 2004). In contrast, HSP90 and other chaperonins are required for proper folding and function of mammalian AhR (Petrulis and Perdew, 2002).

Here we have shown that the *C. elegans* AHR-1 transcription complex has important roles in the regulation of URX activity. In the absence of *ahr-1* or *aha-1* function, key sGC genes are not expressed in URX. This does not appear to be an irreparable developmental defect. Rather, it appears that AHR-1 target genes regulate the response of URX to environmental stimuli. Further genetic studies will clarify the roles of the *C. elegans* aryl hydrocarbon receptor complex in this, and perhaps other, behaviors.

Reference

Ausubel FM (1994) Current protocols in molecular biology. New York: John Wiley & Sons.

Barrett DH, Morris RD, Akhtar FZ, Michalek JE (2001) Serum dioxin and cognitive functioning among veterans of Operation Ranch Hand. Neurotoxicology 22:491-502.

Berezikov E, Bargmann CI, Plasterk RH (2004) Homologous gene targeting in Caenorhabditis elegans by biolistic transformation. Nucleic Acids Res 32:e40.

Birnbaum LS, Tuomisto J (2000) Non-carcinogenic effects of TCDD in animals. Food Addit Contam 17:275-288.

Birnby DA, Link EM, Vowels JJ, Tian H, Colacurcio PL, Thomas JH (2000) A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in caenorhabditis elegans. Genetics 155:85-104.

Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77:71-94.

Butler RA, Kelley ML, Powell WH, Hahn ME, Van Beneden RJ (2001) An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, Mya arenaria: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone binding. Gene 278:223-234.

Cheung BH, Arellano-Carbajal F, Rybicki I, de Bono M (2004) Soluble guanylate cyclases act in neurons exposed to the body fluid to promote C. elegans aggregation behavior. Curr Biol 14:1105-1111.

Cheung BH, Cohen M, Rogers C, Albayram O, de Bono M (2005) Experience-dependent modulation of C. elegans behavior by ambient oxygen. Curr Biol 15:905-917.

Coates JC, de Bono M (2002) Antagonistic pathways in neurons exposed to body fluid regulate social feeding in Caenorhabditis elegans. Nature 419:925-929.

Coburn CM, Bargmann CI (1996) A putative cyclic nucleotide-gated channel is required for sensory development and function in C. elegans. Neuron 17:695-706.

de Bono M, Bargmann CI (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. Cell 94:679-689.

de Bono M, Tobin DM, Davis MW, Avery L, Bargmann CI (2002) Social feeding in Caenorhabditis elegans is induced by neurons that detect aversive stimuli. Nature 419:899-903.

Dong W, Teraoka H, Kondo S, Hiraga T (2001) 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. Neurosci Lett 303:169-172.

Duncan DM, Burgess EA, Duncan I (1998) Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev 12:1290-1303.

Epstein HF, Shakes DC, American Society for Cell Biology. (1995) Caenorhabditis elegans : modern biological analysis of an organism. San Diego: Academic Press.

Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, Bargmann CI (2004) Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. Nature 430:317-322.

Gu YZ, Hogenesch JB, Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol 40:519-561.

Hays LE, Carpenter CD, Petersen SL (2002) Evidence that GABAergic neurons in the preoptic area of the rat brain are targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin during development. Environ Health Perspect 110 Suppl 3:369-376.

Huang X, Powell-Coffman JA, Jin Y (2004) 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. Development 131:819-828.

Jiang H, Guo R, Powell-Coffman JA (2001) The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl Acad Sci U S A 98:7916-7921.

Kim K, Li C (2004) Expression and regulation of an FMRFamide-related neuropeptide gene family in Caenorhabditis elegans. J Comp Neurol 475:540-550.

Komatsu H, Mori I, Rhee JS, Akaike N, Ohshima Y (1996) Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in C. elegans. Neuron 17:707-718.

Morton DB, Hudson ML, Waters E, O'Shea M (1999) Soluble guanylyl cyclases in Caenorhabditis elegans: NO is not the answer. Curr Biol 9:R546-547.

Pelclova D, Fenclova Z, Dlaskova Z, Urban P, Lukas E, Prochazka B, Rappe C, Preiss J, Kocan A, Vejlupkova J (2001) Biochemical, neuropsychological, and neurological abnormalities following 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure. Arch Environ Health 56:493-500.

Petrulis JR, Perdew GH (2002) The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chem Biol Interact 141:25-40.

Pongratz I, Mason GG, Poellinger L (1992) Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. J Biol Chem 267:13728-13734.

Powell-Coffman JA, Bradfield CA, Wood WB (1998) Caenorhabditis elegans orthologs of the aryl hydrocarbon receptor and its heterodimerization partner the aryl hydrocarbon receptor nuclear translocator. Proc Natl Acad Sci U S A 95:2844-2849.

Praitis V, Casey E, Collar D, Austin J (2001) Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. Genetics 157:1217-1226.

Qin H, Powell-Coffman JA (2004) The Caenorhabditis elegans aryl hydrocarbon receptor, AHR-1, regulates neuronal development. Dev Biol 270:64-75.

Rogers C, Reale V, Kim K, Chatwin H, Li C, Evans P, de Bono M (2003) Inhibition of Caenorhabditis elegans social feeding by FMRFamide-related peptide activation of NPR-1. Nat Neurosci 6:1178-1185.

Stringham EG, Dixon DK, Jones D, Candido EP (1992) Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans. Mol Biol Cell 3:221-233.

Wang Q, Wadsworth WG (2002) The C domain of netrin UNC-6 silences calcium/calmodulin-dependent protein kinase- and diacylglycerol-dependent axon branching in Caenorhabditis elegans. J Neurosci 22:2274-2282.

White JG, Southgate E, Thomson JN, Brenner S (1986) The structure of the nervous system of the nematode C. elegans. Philos Trans R Soc Lond B Biol Sci 314:1-340.

Table 1: AHR-1 positively regulates expression of soluble guanylate cyclase genes in the URX neurons: analysis of transgenic animals carrying GFP reporters in extrachromosomal arrays

Extrachromosomal Array Transgenes	Genetic Background	GFP expression in URX cells					
		% Very Strong	% Strong	% Dim	% very Dim	% N. D.	n
**Pgcy-32:*GFP	Wild Type	19	73	0	2	6	126
	ahr-1 (ia03)	0	0	0	34	66	111
*Pgcy-34:*GFP	Wild Type	100	0	0	0	0	132
	ahr-1 (ia03)	0	43	31	18	8	123
*Pgcy-35:*GFP	Wild Type	99	0	0	0	1	126
	ahr-1 (ia03)	0	12	47	39	2	132
*Pgcy-36:*GFP	Wild Type	95	2	0	1	2	131
	ahr-1 (ia03)	53	40	4	2	1	126
*Pgcy-37:*GFP	Wild Type	100	0	0	0	0	121
	ahr-1 (ia03)	61	36	2	2	0	125

*(Qin & Powell-Coffman, 2004). n=number scored.

GFP expression: Very Strong = Cell body and axon fluoresced brightly; Strong = GFP fluorescence is easily detectable in both cell body and axon; Dim = GFP fluorescence is detectable in the cell body, but the expression in the axon is faint or not detectable; Very Dim = GFP fluorescence is faint in the cell body, and the expression in the axon is not detectable; N.D. = not detectable.

Table 2: Regulation of soluble guanylate cyclase gene expression in the URX neurons, as assayed by GFP reporters integrated into the genome

Integrated Transgenes	Genetic Background	GFP expression in URX cells					
		% Very Strong	% Strong	% Dim	% very Dim	% N. D.	n
	Wild Type	0	106	1	0	0	107
	ahr-1 (ia03)	0	0	0	2	101	103
*Pgcy-32:*GFP	*aha-1 (ia01)**	0	0	0	64	47	111
	daf-21(p673)	0	62	0	0	0	62
	daf-21(nr2081)	0	72	0	0	0	72
	Wild Type	0	119	0	0	0	119
	ahr-1 (ia03)	0	0	0	126	0	126
*Pgcy-34:*GFP	*aha-1 (ia01)**	0	0	16	88	0	104
	daf-21(p673)	0	105	0	0	0	105
	daf-21(nr2081)	0	61	0	0	0	61
	Wild Type	0	102	0	0	0	102
*Pgcy-35:*GFP	*ahr-1 (ia03)*	0	0	2	106	0	108
	*aha-1 (ia01)**	0	0	1	100	6	107
	Wild Type	112	0	0	0	0	112
*mPgcy-35:*GFP	*ahr-1 (ia03)*	0	0	106	0	0	106
	*aha-1 (ia01)**	0	1	114	0	0	115
	Wild Type	0	109	0	0	0	109
*Pgcy-36:*GFP	*ahr-1 (ia03)*	0	0	106	0	0	106
	*aha-1 (ia01)**	0	0	105	0	0	105
	Wild Type	0	107	6	0	0	113
*mPgcy-36:*GFP	*ahr-1 (ia03)*	0	0	110	8	0	118
	*aha-1 (ia01)**	0	0	102	11	0	113
	Wild Type	113	0	0	0	0	113
*Pgcy-37:*GFP	*ahr-1 (ia03)*	0	118	5	0	0	123
	*aha-1 (ia01)**	46	68	0	0	0	114
	daf-21(nr2081)	60	0	0	0	0	60

* These animals are mosaic for *aha-1* expression. They are homozygous for the *aha-1(ia01)* mutation, but carry a *cky-1:aha-1* transgene that directs expression of AHA-1 in nonneuronal cells of the pharynx.

The *mPgcy-35*:GFP and *mPgcy-36*:GFP constructs have a point mutation in the putative AHR-1 halfsite.

Figure Legends

Figure 1 AHR-1 functions in URX neurons to promote aggregation behavior.

The requirements for *ahr-1* and *aha-1* in aggregation behavior were examined. Two characteristic behaviors are quantitated: collection of animals in groups of 3 or more (**A, C**) and bordering (**B, D**). In **A.** and **B.**, wild type alleles of *ahr-1* or *aha-1* are indicated by +. Some aggregation behavior can be restored to *ahr-1* mutant animals by introduction of an *ahr-1* minigene (*Pahr-1:ahr-1*) or by *flp-8:ahr-1*, which drives expression of *ahr-1* in URXR, URXL, AUA, and PVM neurons (**C., D.**) *ahr-1(ia03), ahr-1(ju145), aha-1(ia01), npr-1(ky13)*, and *npr-1(ad609)* are all loss-of-function alleles.

Figure 2 Induction of *ahr-1* expression from a heat shock promoter in the last larval stage is sufficient to promote aggregation behavior in *ahr-1, npr-1* double mutant young adults.

URX neurons are born during embryogenesis, and aggregation behavior is evident in *npr-1*-deficient animals by the L2 larval stage. Two *npr-1* mutant alleles were assayed (*ky13* and *ad609*). Grouping behavior and bordering behavior were assayed for each experimental condition.

Figure 3 Potential DNA binding sites for the AHR-1:AHA-1 complex in the 5' regulatory regions of soluble guanylate cyclase genes.

The DNA recognition sites for mammalian AhR and ARNT are shown. *C. elegans* AHR-1 and AHA-1 also interact to bind this consensus sequence (termed XRE). Putative XREs were identified in all five sGC genes, and the positions of these sequences relative to the start codons are shown. Underlined bases identify the point mutations that were introduced to the *gcy-35* and *gcy-36* reporters.

Figure 4 Model for AHR-1 function in the URX neurons.

The URX neurons play a central role in hyperoxia avoidance and aggregation behavior. NPR-1 activity inhibits URX depolarization and aggregation behavior. The TAX-2 / TAX-4 cGMP gated ion channel promotes URX depolarization. GCY-35/GCY-36 guanylate cyclase complex responds to ambient oxygen and produces cGMP, thus promoting URX depolarization. The AHR-1:AHA-1 transcription complex functions in these neurons and induces the expression of multiple sGC genes, including *gcy-35* and *gcy-36*. Since mutation of the putative XRE in gcy-35 and gcy-36 does not abrogate regulation by AHR-1 and AHA-1, we postulate that AHR-1 acts through other unidentified factors to control *gcy-35* and *gcy-36* expression.

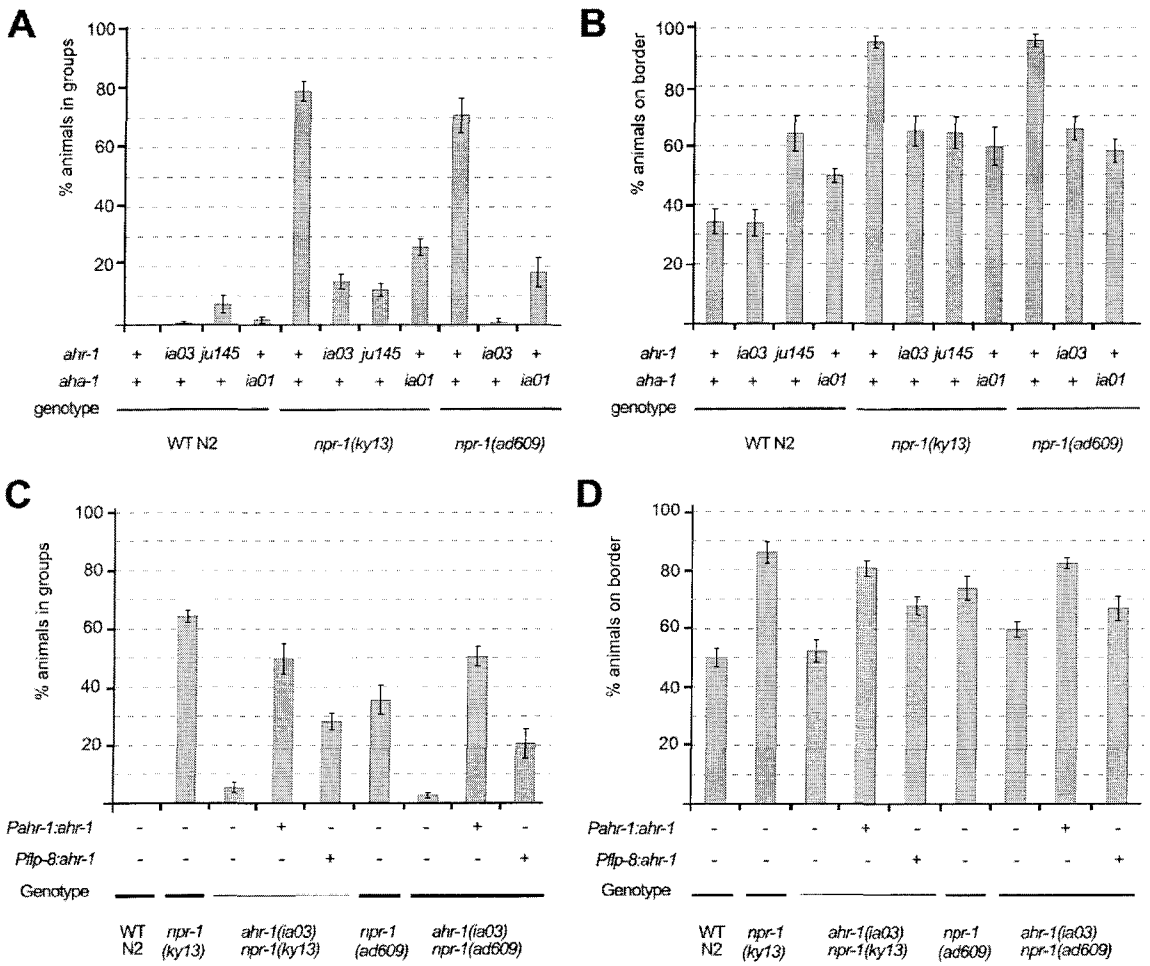
A
% animals in groups
100
80
60
40
20
ahr-1 + ia03 ju145 + + ia03 ju145 + + ia03 +
aha-1 + + + ia01 + + + ia01 + + ia01
genotype
WT N2
npr-1(ky13)
npr-1(ad609)
B
% animals on border
100
80
60
40
20
ahr-1 + ia03 ju145 + + ia03 ju145 + + ia03 +
aha-1 + + + ia01 + + + ia01 + + ia01
genotype
WT N2
npr-1(ky13)
npr-1(ad609)
C
% animals in groups
100
80
60
40
20
Pahr-1:ahr-1 - - - + - - - + -
Pflp-8:ahr-1 - - - - + - - - +
Genotype
WT N2
npr-1 (ky13)
ahr-1(ia03) npr-1(ky13)
npr-1 (ad609)
ahr-1(ia03) npr-1(ad609)
D
% animals on border
100
80
60
40
20
Pahr-1:ahr-1 - - - + - - - + -
Pflp-8:ahr-1 - - - - + - - - +
Genotype
WT N2
npr-1 (ky13)
ahr-1(ia03) npr-1(ky13)
npr-1 (ad609)
ahr-1(ia03) npr-1(ad609)

Figure 1

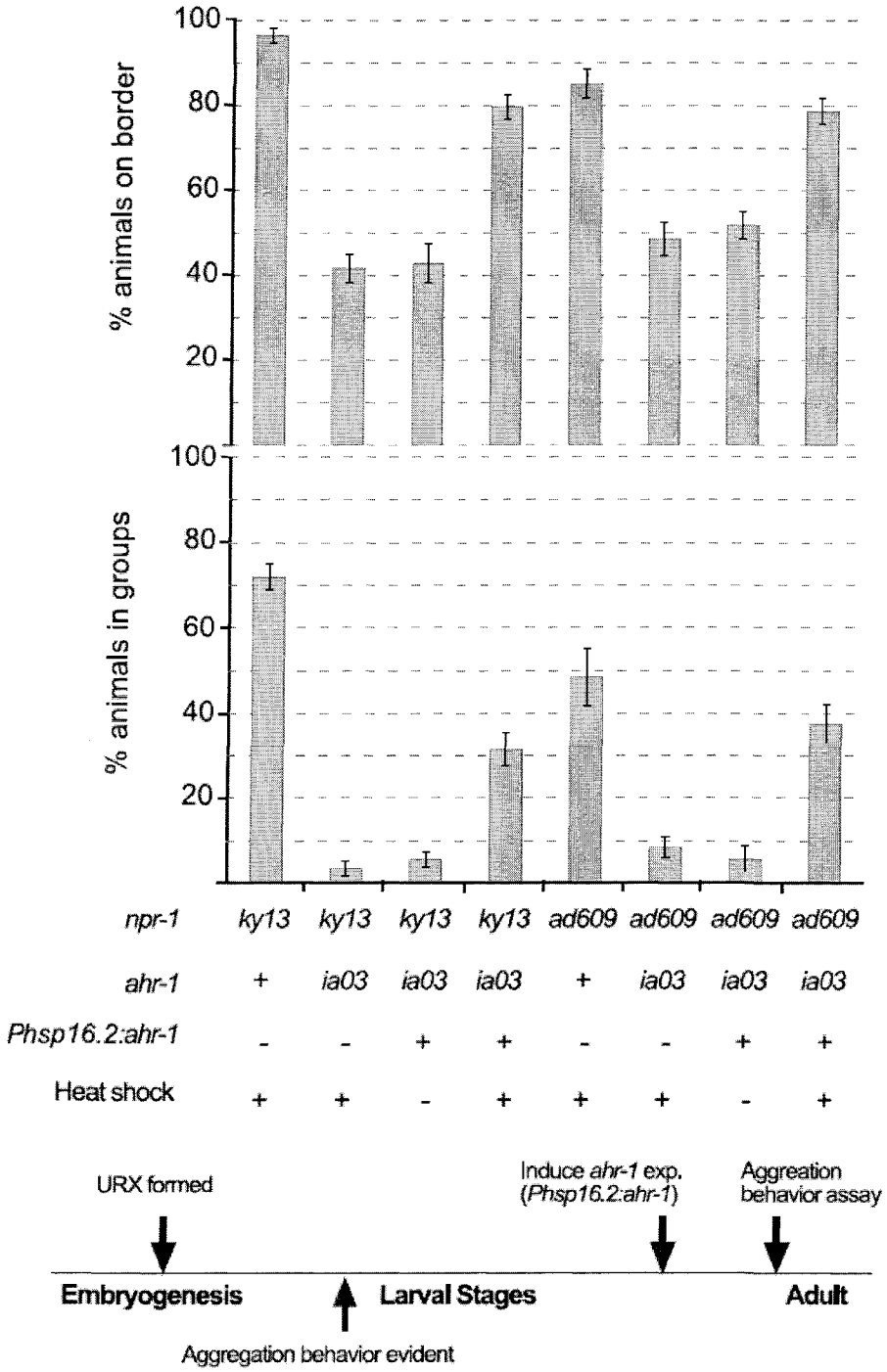
100
80
60
40
20
% animals on border
100
80
60
40
20
% animals in groups
npr-1 ky13 ky13 ky13 ky13 ad609 ad609 ad609 ad609
ahr-1 + ia03 ia03 ia03 + ia03 ia03 ia03
Phsp16.2:ahr-1 - - + + - - + +
Heat shock + + - + + + - +
URX formed
Induce ahr-1 exp. (Phsp16.2:ahr-1)
Aggreation behavior assay
Embryogenesis
Larval Stages
Adult
Aggregation behavior evident

Figure 2

AHR	KNGC
ARNT	GTG
AHR-1:AHA-1	KNGCGTG
gcy-32 (-616bp)	aaTGGCGTGgc
(-157bp)	gaTGGCGTGcg
(-80bp)	gaTGGCGTGtc
gcy-34 (-82bp)	aaTCGCGTGcc
gcy-35 (-111bp)	caTTGCGTGtc
gcy-36 (-263bp)	caTTGCGTGtc
gcy-37 (-126bp)	ccTTGCGTGtc

Figure 3

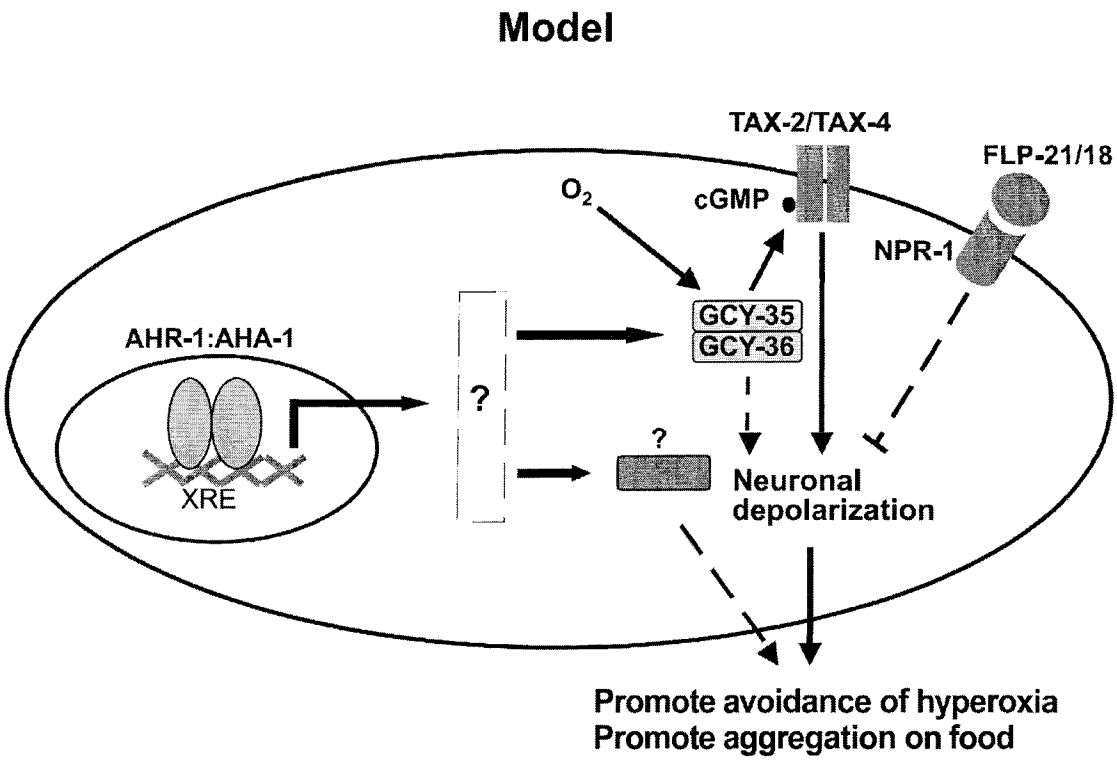
Model
TAX-2/TAX-4
FLP-21/18
O_2
cGMP
NPR-1
GCY-35
GCY-36
AHR-1:AHA-1
?
?
XRE
Neuronal depolarization
Promote avoidance of hyperoxia
Promote aggregation on food

Figure 4

CHAPTER 4 GENERAL CONCLUSIONS

Summarization

In this dissertation, I analyze the endogenous function of *C. elegans* aryl hydrocarbon receptor, *ahr-1*. The major findings include: (1) *ahr-1* is expressed primarily in the neural system, as indicated by the expression pattern of *ahr-1*:GFP reporters. I have identified all 28 neurons that express *ahr-1* in early L1 larvae. These neurons belong to different neuronal subtypes. (2) Normal *ahr-1* function is required to regulate neuronal development as evidenced by aberrant cell migration, axon path finding and gene expression in *ahr-1*-deficient animals. (3) I identified a transcription cascade in which UNC-86 positively regulates the expression of *ahr-1* in URX neurons and AHR-1 positively regulates the expression of *npr-1* and several soluble guanylate cyclate genes. (4) AHR-1 functions acutely in neurons to regulate their physiological function and promote aggregation behavior.

AHR-1 regulates neuronal development

A young adult hermaphrodite is composed of 959 somatic nuclei, including 302 neurons (White et al., 1986). We created two *ahr-1:GFP* reporters and analyzed the expression patterns of these reporters (Chapter 2). As indicated by *ahr-1:GFP* reporters, *ahr-1* is expressed primarily in the neural system of *C. elegans*. The expression is limited to a small group of neural cells. We have identified all the cells that express *ahr-1:GFP* in L1 larvae and these cells include 28 neurons and 2 socket cells. Some of these neurons are generated postembryonically in early L1 larvae. In newly hatched larvae, the QR neuroblast cell is originally located at the posterior body, and it migrates from posterior to anterior. QR divides during the migration and its descendant cells differentiate into 3 neurons, AVM, AQR and SDQR. *ahr-1:GFP* does not express in QR. However, the GFP is detected in all of the QR's descendant cells during their migration and differentiation. Similar expression patterns were observed in QL and T cell lineages.

Phenotypic analyses of *ahr-1* loss-of-function mutants suggest that normal *ahr-1* function is required for the appropriate development of AVM, AQR and SDQR neurons, as evidenced by aberrant cell migration, axon pathfinding and gene expression. The neuronal development function of AHR-1 is not restricted to the QR cell lineage or to any specific neuron subtype. PLML and PLMR are two touch neurons in the tail and are generated during embryonic development. In *ahr-1*-deficient animals, PLML and PLMR also exhibit cell migration and axon pathfinding defects. In addition to these defects, some *ahr-1*-deficient animals have one or more extra cells that express a PLM cell type specific GFP marker. This suggests that *ahr-1* function is required for programmed cell death or lack of *ahr-1* function causes other cells to adopt PLM cell fate.

The requirement for *ahr-1* in neural development was further supported by an independent study. Elaborate studies have demonstrated that AHR-1 determined the cell fate specification of RME GABAergic neurons (Huang et al., 2004). In *ahr-1*-deficient animals, RMEL and RMER neurons adopt the cell fates of RMED and RMEV. In contract, ectopically expressing *ahr-1* in RMED and RMEV neurons transforms these neurons into RMEL and RMER like neurons.

Like its mammalian cognate, AHR-1 can form a protein complex with AHA-1 (*C. elegans* ARNT homolog) and bind to an XRE specifically *in vitro*. This suggests that AHR-1 forms a transcription complex *in vivo* with AHA-1 to regulate the expression of developmental important genes. This hypothesis predicts that AHA-1 normal function is also required to regulate neuronal development. To test this hypothesis, we analyzed *aha-1*-deficient worms and mosaic worms that lack AHA-1 function in most of the non-pharyngeal neurons. These worms exhibit SDQR migration defect and RMEL/D specification defect similar to those of *ahr-1*-deficient worms (Huang et al., 2004; Qin and Powell-Coffman, 2004). Thus these genetic data support the hypothesis that AHR-1 functions in a transcription complex with AHA-1 to regulate neuronal development.

During the neuronal development, transcription factors function in transcription cascades to specify the cell fate of neurons. We reported a transcription cascade in which UNC-86 acts upstream of *ahr-1* to regulate the expression of *npr-1* and multiple soluble guanylate cyclase genes in URXL and URXR neurons. These results and the generated reagents provide the foundation for a GFP based genetic screening to identify regulators for *ahr-1* signaling. The recently identified RNAi sensitive mutant strains make it feasible to efficiently knock down gene activity in neurons by feeding the worms with bacteria that express double strand mRNA (Sieburth et al., 2005). Combining traditional forward genetic screening with RNAi screening will facilitate the process of identifying *ahr-1* regulators.

To understand the mechanism by which AHR-1 regulates SDQR development, we created double mutant of *ahr-1* with genes in known signaling pathways that regulate or might regulate SDQR migration, including UNC-6/Netrin, SLT-1/SLIT and UNC-129/TGF-β. All double mutants significantly enhance the SDQR migration defects of each single mutant. These data suggest two nonexclusive possibilities: 1) AHR-1 might mediate response to one or more unidentified dorsal-ventral SDQR guidance signal. 2) AHR-1 might mediate the response to multiple SDQR dorsal-ventral guidance cues. Considering the high penetrance of SDQR migration defects in *ahr-1* (53%) and *unc-6* (74%) mutants, it is more likely that AHR-1 mediates the cellular response to multiple guidance cues. Many cytoplasmic molecules that mediate guidance cue signaling for cell migration and axon pathfinding have been identified, including members of the Rho family GTPases, GEFs (UNC-73/Trio), UNC-34/Enabled, UNC-115 (a putative active binding protein), UNC-44/Ankyrin, SRC-1 (a non-receptor protein tyrosine kinase) and others (Otsuka et al., 1995; Steven et al., 1998; Montell, 1999; Wu et al., 2002; Yu et al., 2002; Fukata et al., 2003; Gitai et al., 2003; Itoh et al., 2005; Shakir et al., 2006). However, none of the cytoplasmic molecules that regulate SDQR cell migration and/or axon pathfinding have been identified. Identifying AHR-1 target genes that function in the

SDQR neuron to mediate the response to guidance signaling will provide insights to understand how the receptors transfer guidance signals to the cytoskeleton and achieve the migration events.

AHR-1 regulates aggregation behavior

ahr-1-deficient animals are viable and do not exhibit dramatic behavioral defects. These worms can move, mate well and respond to many chemicals as WT worms (unpublished data of JAPC lab). However, careful behavior assays demonstrate that normal *ahr-1* function is required to promote the aggregation feeding behavior of *npr-1* mutants (Chapter 3). *C. elegans* wild type strain N2 feeds solitarily on bacteria food, while some strains isolated from the wild feed in groups. This behavioral variance is associated with two NPR-1 protein isoforms. Strains carrying high activity NPR-1 isoform feed individually, while strains carrying low NPR-1 activity isoform feed in groups. *npr-1* loss-of-function mutants exhibit stronger aggregation behavior than "social" strains (de Bono and Bargmann, 1998). *ahr-1*-deficient worms are solitary feeders, like the wild type N2 strain. However, *ahr-1* loss-of-function mutants suppress the aggregation behavior of *npr-1* mutants. Aggregation behavior is finely regulated by gene networks that function in complicated neuronal circuits. Several groups of neurons have been identified that control aggregation behavior (Coates and de Bono, 2002; de Bono et al., 2002). *ahr-1* is expressed in four of these neurons, URXL, URXR, AQR and PQR. To test if *ahr-1*'s function in URX (URXL and URXR) neurons is sufficient to promote aggregation behavior, we created mosaic transgenic worms and showed that the expression of *ahr-1* in only four neurons, URXL, URXR, AUA and PVM, using the *flp-8* promoter is sufficient to restore aggregation behavior to *ahr-1, npr-1* double mutants. 5% of *ahr-1 (ia03); npr-1 (ky13)* double mutants feed in groups. Introduction of the *flp-8*::*ahr-1* transgene increases aggregation behavior over 5-fold (28% of the animals feed in groups). Touch neuron PVM is not required for aggregation behavior. *ahr-1*:GFP does not express in interneuron AUA. Thus, we favor a model that AHR-1 functions in URX neurons to regulate aggregation behavior.

Currently, *flp-8* is the only promoter that is appropriate for this study. The expression of *gcy-32* and other soluble guanylate cyclase genes that express in URX is controlled by AHR-1 transcription factor. Using these promoters to drive the expression of transgenes in *ahr-1*-deficient animal is infeasible. Other URX cell type specific genes have broad expression patterns that could compromise the results.

URXL and URXR neurons are generated in the embryo. It is not clear whether *ahr-1:*GFP is expressed in their progenitor cells and during the differentiation of these neurons. However, *ahr-1:*GFP is detectable in URX neurons by the late embryonic stage, and the expression is maintained at high level until the worms die. This type of expression pattern raises two non-exclusive models to explain how AHR-1 regulates aggregation behavior: 1) AHR-1's function is required during critical development period to regulate the development of the neurons. 2) AHR-1's function is required acutely to regulate the physiological function of the neurons.

Unlike AVM and SDQR neurons, URX neurons have no obvious morphological defects in *ahr-1*-deficient worms. In *ahr-1(ia03)* mutants, URX neurons are generated and their axons appear as normal. We analyzed AHR-1's development roles in URX neurons by testing gene expression. Normal *ahr-1* function is not required for the expression of a panneuron GFP marker, nor a cell type specific GFP marker *egl-2*. However, the expression of several cell type specific genes, including *npr-1* and several soluable guanylate cyclate genes depends on AHR-1 function. These data suggest that AHR-1 function is required to regulate a subset of development program of URX neurons. This developmental function might or might not contribute to the regulation of aggregation behavior.

To test the hypothesis that AHR-1 regulates the physiological function of URX neurons to promote aggregation behavior, we used a heat shock protein promoter *hsp16.2* to drive the expression of AHR-1 temporally. Inducing AHR-1 expression by thirty minutes of heat shock after URX neurons have fully developed is sufficient to restore aggregation

behavior to *ahr-1, npr-1* double mutants. This clearly demonstrates that AHR-1 can facilitate aggregation behavior by regulating the physiological function of neurons. AHR-1:AHA-1 transcription complex controls the expression of *gcy-35* and *gcy-36*, two key regulators of *npr-1*-mediated aggregation behavior. GCY-35 and GCY-36 function as an α/β soluble guanylate cyclate to induce aggregation behavior (Cheung et al., 2004; Gray et al., 2004). High guanylate cyclate activity facilitates the worms to avoid hyperoxia environment and induces aggregation behavior (Gray et al., 2004). Oxygen binds GCY-35 *in vitro* and the enzyme activity of Drosophila atypical guanylate cyclates can be regulated acutely by oxygen concentration in COS-7 cells (Gray et al., 2004; Morton, 2004). Low oxygen (O_2) concentration suppresses *npr-1*-mediated aggregation behavior in a quantitive manner. Switching the worms from low oxygen condition back to normal plates releases the *npr-1*-mediated aggregation behavior within 3 minutes (Gray et al., 2004). Thus, oxygen may acutely regulate the guanylate cyclate activity of GCY-35 and GCY-36, and the generant cGMP signal regulates the neuronal activity of URX to promote aggregation behavior. These evidences suggest that AHR-1 controls the expression of *gcy-35* and *gcy-36* genes and thereby acutely regulates URX neuronal activity to promote aggregation behavior. However, AHR-1 might also regulate the expression of other genes that have important roles in regulating aggregation behavior. Supporting this hypothesis, using *flp-8* promoter to express *gcy-35* and/or *gcy-36* in URX neurons can not restore aggregation behavior to *ahr-1, npr-1* double mutants. Each *gcy-32* or *gcy-34* single mutant does not suppress *npr-1*-mediated aggregation behavior (Cheung et al., 2004). However, a recent study have reported that *gcy-32, gcy-34* double mutant suppress *npr-1*-mediated aggregation behavior (Cheung et al., 2005). Fully understanding how AHR-1 regulates aggregation behavior requires identifying AHR-1 dependent genes in URX neurons.

Significance of this study

The work reported in this dissertation initiates the studies of AHR endogenous neural functions in a powerful genetic model system *C. elegans*. The results from this study

provide the framework for understanding AHR endogenous functions in neuronal systems. My work has revealed that AHR-1 plays important roles in regulating both neuronal development and neuron activity. A study by another group focused on a group of GABAergic neurons, and they conclude that AHR-1 controls the cell fate specification of RME neurons (Huang et al., 2004). A recent publication demonstrates that *Drosophila* AHR/*Spineless* determines the cell fate specification of R7 photoreceptor cells in the ommatidia (Wernet et al., 2006). These findings suggest that ancestral AHR has a conserved role in regulating neuronal development. Toxicological studies in vertebrates have shown that AHR functions in neural system to mediate the neural toxic effect of TCDD presumably by interfering with neural development and the physiological function of the brain. Thus, vertebrate AHR might have conserved endogenous functions in neural system. Studying AHR-1 function and regulation will provide significant clues that will help us to fully understand the AHR functions in human being and other experimental organisms.

Reference

Cheung BH, Arellano-Carbajal F, Rybicki I, de Bono M (2004) Soluble guanylate cyclases act in neurons exposed to the body fluid to promote C. elegans aggregation behavior. Curr Biol 14:1105-1111.

Cheung BH, Cohen M, Rogers C, Albayram O, de Bono M (2005) Experience-dependent modulation of C. elegans behavior by ambient oxygen. Curr Biol 15:905-917.

Coates JC, de Bono M (2002) Antagonistic pathways in neurons exposed to body fluid regulate social feeding in Caenorhabditis elegans. Nature 419:925-929.

de Bono M, Bargmann CI (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. Cell 94:679-689.

de Bono M, Tobin DM, Davis MW, Avery L, Bargmann CI (2002) Social feeding in Caenorhabditis elegans is induced by neurons that detect aversive stimuli. Nature 419:899-903.

Duncan DM, Burgess EA, Duncan I (1998) Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev 12:1290-1303.

Fukata M, Nakagawa M, Kaibuchi K (2003) Roles of Rho-family GTPases in cell polarisation and directional migration. Curr Opin Cell Biol 15:590-597.

Gitai Z, Yu TW, Lundquist EA, Tessier-Lavigne M, Bargmann CI (2003) The netrin receptor UNC-40/DCC stimulates axon attraction and outgrowth through enabled and, in parallel, Rac and UNC-115/AbLIM. Neuron 37:53-65.

Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, Bargmann CI (2004) Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. Nature 430:317-322.

Huang X, Powell-Coffman JA, Jin Y (2004) 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. Development 131:819-828.

Itoh B, Hirose T, Takata N, Nishiwaki K, Koga M, Ohshima Y, Okada M (2005) SRC-1, a non-receptor type of protein tyrosine kinase, controls the direction of cell and growth cone migration in C. elegans. Development 132:5161-5172.

Montell DJ (1999) The genetics of cell migration in Drosophila melanogaster and Caenorhabditis elegans development. Development 126:3035-3046.

Morton DB (2004) Atypical soluble guanylyl cyclases in Drosophila can function as molecular oxygen sensors. J Biol Chem 279:50651-50653.

Otsuka AJ, Franco R, Yang B, Shim KH, Tang LZ, Zhang YY, Boontrakulpoontawee P, Jeyaprakash A, Hedgecock E, Wheaton VI, et al. (1995) An ankyrin-related gene (unc-44) is necessary for proper axonal guidance in Caenorhabditis elegans. J Cell Biol 129:1081-1092.

Qin H, Powell-Coffman JA (2004) The Caenorhabditis elegans aryl hydrocarbon receptor, AHR-1, regulates neuronal development. Dev Biol 270:64-75.

Shakir MA, Gill JS, Lundquist EA (2006) Interactions of UNC-34 Enabled With Rac GTPases and the NIK Kinase MIG-15 in Caenorhabditis elegans Axon Pathfinding and Neuronal Migration. Genetics 172:893-913.

Sieburth D, Ch'ng Q, Dybbs M, Tavazoie M, Kennedy S, Wang D, Dupuy D, Rual JF, Hill DE, Vidal M, Ruvkun G, Kaplan JM (2005) Systematic analysis of genes required for synapse structure and function. Nature 436:510-517.

Steven R, Kubiseski TJ, Zheng H, Kulkarni S, Mancillas J, Ruiz Morales A, Hogue CW, Pawson T, Culotti J (1998) UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in C. elegans. Cell 92:785-795.

Wernet MF, Mazzoni EO, Celik A, Duncan DM, Duncan I, Desplan C (2006) Stochastic spineless expression creates the retinal mosaic for colour vision. Nature 440:174-180.

White JG, Southgate E, Thomson JN, Brenner S (1986) The structure of the nervous system of the nematode C. elegans. Philos Trans R Soc Lond B Biol Sci 314:1-340.

Wu YC, Cheng TW, Lee MC, Weng NY (2002) Distinct rac activation pathways control Caenorhabditis elegans cell migration and axon outgrowth. Dev Biol 250:145-155.

Yu TW, Hao JC, Lim W, Tessier-Lavigne M, Bargmann CI (2002) Shared receptors in axon guidance: SAX-3/Robo signals via UNC-34/Enabled and a Netrin-independent UNC-40/DCC function. Nat Neurosci 5:1147-1154.

ACKNOWLEDGEMENTS

I thank my supervisor Dr. Jo Anne Powell-Coffman for her teaching, guidance, encouragement, and generous support through my graduate career at Iowa State University.

I am grateful to Dr. Jack R. Girton, Dr. Kristen M. Johansen, Dr. James M. Reecy, and Dr. Donald S. Sakaguchi for serving on my committee and giving advice and help.

I am indebted to all members of the Powell-Coffman lab and Coffman lab for their assistance in daily bench work, comments on the project, and friendship.

I thank Robert Doyle in the Roy J. Carver Center for Biological Scanning Probe and Laser-based Microscopy at ISU for his expert assistance on confocal microscopy imaging. I thank the ISU plant transformation facility and Mr. Kuang, Xianyan for helping us develop a micropartical bombardment protocol to create integrated *C. elegans* transgenic lines. I thank Dr. Thomas Peterson for sharing the Biolistic PDS-1000/He with us.

I thank Linda Wild for her friendship and assistance.